INDUCTION AND EXPRESSION OF DELAYED HYPERSENSITIVITY IN THE SMALL INTESTINE

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SUMMARY

Cell mediated immunity to dietary proteins may cause small intestinal injury and malabsorption and the work of this thesis was designed to investigate in an animal model, the factors governing the induction and expression of intestinal CMI.

Increases in crypt cell production rate and intraepithelial lymphocyte count were found to be reliable indices of mucosal CMI during the GvHR in neonatal and adult mice. These were direct consequences of the CMI response and evidence was obtained that cytotoxicity did not account for the mucosal changes. I propose that soluble mediators released by T cells activated in the mucosa or gut associated lymphoid tissues are the factors responsible.

In mice fed ovalbumin, CMI was identified in the mucosa using the CCPR and IEL count and in the GALT by a lymph node lymphocyte migration inhibition assay. This test was shown to correlate with systemic CMI in parenterally immunised mice. Normal mice fed OVA did not develop intestinal CMI but became systemically tolerant to OVA. Mice given cyclophosphamide before feeding OVA, developed CMI in the GALT and mucosa, and the induction of systemic tolerance was also inhibited by CY. CMI in the GALT developed rapidly, was recalled by oral challenge with OVA and systemic immunity was not detected in fed mice. Systemic CMI was more readily suppressed by feeding OVA than humoral immunity and the tolerance of CMI was particularly sensitive to CY pretreatment. In addition,

serum from OVA fed mice suppressed the CMI response of recipients but not their antibody response. These findings indicated that, after feeding proteins, systemic and intestinal CMI responses are regulated by a CY-sensitive mechanism and I postulate that this system is activated by intestinally derived protein moieties.

Detailed studies of the mucosa and GALT of CY treated mice revealed minor abnormalities which were related to alterations in crypt cell turnover. The antigen-processing structures of the Peyer's Patches were not damaged by CY and intestinal uptake of OVA was not altered quantitatively or qualitatively by CY. These findings, and published evidence, indicate that CY had altered intestinal immunity by its action on suppressor cells.

I have shown therefore that it is possible to measure intestinal CMI both in the mucosa and GALT. Local responses to fed proteins do not occur in normal animals probably due to the action of an intestinal suppressor cell system. Abrogation of this system allows local CMI to develop and mucosal damage may occur as a result of the action of lymphokines produced during these responses. Defective suppressor cell responses to fed antigen may underly enteropathies associated with food hypersensitivity.

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Publications

Work described in this thesis is included in the following publications.

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Abbreviations used in the text

α	anti-
Ab	antibody
Ag	antigen
B lymphocyte	bone marrow derived lymphocyte
BCG	Bacille Calmette-Guérin
BSA	bovine serum albumin
C'	complement
CO_2	carbon dioxide
CCPR	crypt cell production rate
CFA	Complete Freund's Adjuvant
CMI	Cell mediated immunity
Con A	concanavalin A
CY	cyclophosphamide
DTH	delayed-type hypersensitivity
DNA	deoxyribonucleic acid
DNP-BGG	dinitrophenyl bovine gamma globulin
EM	electron microscope
F_1	first generation
Fab_2	pepsin cleaved fragment of immunoglobulin
FCS	foetal calf serum
FITC	fluorescein isothiocyanate
GALT	gut-associated lymphoid tissues
GvHD	graft-versus-host disease
GvHR	graft-versus-host reaction
H-2	histocompatibility locus-2 (mouse)
H&E	haematoxylin and eosin
HSA	human serum albumin

^{3}H-TdR	tritiated thymidine
I-A	subregion of murine major histocompatibility complex
i.d.	intradermal
Ig	immunoglobulin
IEL	intraepithelial lymphocyte
IFA	incomplete Freund's adjuvant
i.p.	intraperitoneal
Lyt	T lymphocyte associated antigen (mouse)
MHC	major histocompatibility complex
M cell	microfold cell
MI	migration index
MIF	macrophage migration inhibitory factor
MLN	mesenteric lymph node
MMC	mucosal mast cell
n	number of
O.D.	optical density
OVA	ovalbumin
Oxaz	oxazolone
p	probability
PFC	plaque-forming cell
PHA	phytohaemagglutinin
PBS	phosphate buffered saline
r	correlation coefficient
s.d.	standard deviation
s.e.m.	standard error of the mean
S.E.M.	scanning electron microscope
sIg	surface immunoglobulin

SRBC	sheep red blood cells
T lymphocyte	thymus derived lymphocyte
T_{DTH}	T lymphocyte responsible for DTH
T_H	helper T lymphocyte
T_S	suppressor T lymphocyte
TDL	thoracic duct lymph
T.E.M.	transmission electron microscope
Thy 1.2	T lymphocyte-specific alloantigen (mouse)
T.spiralis	Trichinella spiralis

<u>length</u>

cm	centimetre
mm	millimetre
µm	micrometre
nm	nanometre

<u>volume</u>

l	litre
ml	millilitre
µl	microlitre

<u>weight</u>

kg	kilogram
g	gram
mg	milligram
µg	microgram

<u>concentration</u>

M	molar
mM	millimolar
N	normal

Miscellaneous

g	gravitational acceleration
$\log_{10}$	logarithm to the base 10
pH	reciprocal $\log_{10}$ hydrogen ion concentration

Symbols

<	less than
⩽	less than or equal to
>	greater than
≒	approximately equal to
/	per

CHAPTER 1

INTRODUCTION

The normal small intestine has several different functions of which the principal are the digestion and absorption of foods. However, the intestine also acts as a "barrier", reducing or preventing access to the body of potentially harmful organisms and toxins. The factors responsible for this protective function include intestinal motility, production of mucus and other secretions and the ability to mount specific immune responses. The morphology of the small intestinal mucosa reflects in part these functions. Thus, the epithelium includes goblet cells, Paneth cells and enteroendocrine cells while large numbers of lymphoid cells are found both within the epithelium and in the underlying lamina propria. The intimate relationship between epithelial and lymphoid elements is further underlined by the fact that under certain circumstances, an immune response in the mucosa may damage the functional compartment of the gut. In this way, local immune reactions may induce abnormalities of both structure and function (Ferguson 1976).

Several small intestinal diseases are associated with disordered mucosal architecture, including coeliac disease, cows milk protein intolerance, tropical sprue and parasitic infestations. In many of these conditions, an immune response to an enteric antigen has been implicated in the development of intestinal injury, and it is likely that this immunity is taking place in the mucosa itself. The lymphoid cells of the mucosa include both thymus dependent

and independent lymphocytes as well as large numbers of macrophages, polymorphs, eosinophils and mast cells. The capacity therefore exists in the intestinal mucosa for the expression of antibody dependent, reaginic and cell-mediated immunity. (Parrott 1976).

Each of these forms of immunity can be identified during local responses to enteric antigens and the production of secretory antibodies is now well documented. However, in comparison to humoral immunity at secretory surfaces, mucosal cell mediated immunity has received little attention. Very little is known of the factors governing the induction of CMI in the small intestine and of the interactions between the effector limb of mucosal CMI and the structure and function of the intestinal mucosal itself. While villous atrophy occurs during the CMI response involved in allograft rejection and in graft versus host reaction (MacDonald 1976; MacDonald & Ferguson 1977), a possible role for mucosal CMI in the food allergic diseases associated with villous atrophy and malabsorption has only recently been considered.

The overall aim of the work described in this thesis was to study, in an animal model, the factors which govern the induction and expression of intestinal cell mediated immunity to a dietary protein antigen. The following approaches were used to achieve this objective.

1) Reliable parameters of the effector limb of mucosal cell mediated immunity were sought by producing a "classical" form of CMI in the small intestine and following the

consequences in the mucosa. The GvHR, induced in neonatal and adult F_1 mice by the injection of parental lymphocytes, was employed for this purpose. I have examined the hypothesis that the intraepithelial lymphocyte count and crypt cell production rate correlate closely with the mucosal CMI of the GvHR and the relevant experiments and results are presented in Chapters 6 and 7.

2) Since these indices applied to the mucosa itself were necessarily indirect parameters of CMI, a direct, immunological assay for CMI which could be applied to the gut-associated lymphoid tissues was developed. The experiments described in Chapter 8 therefore deal with migration inhibition of lymph node cells in vitro, and this assay was shown to correlate with CMI in vivo.

3) Using these parameters of mucosal and GALT immunity, the induction of local CMI and mucosal injury following oral immunisation with a defined protein antigen was then investigated. These studies are described in Chapters 9 and 10. In these experiments, cyclophosphamide was used to abrogate the state of immunological unresponsiveness which normally results from feeding proteins. In consequence, Chapter 11 describes experiments designed to study the phenomenon of oral tolerance in more detail, while in Chapters 12 and 13, the effects of cyclophosphamide itself on the intestinal mucosa are considered.

CHAPTER 2

T LYMPHOCYTES, CELL MEDIATED IMMUNITY AND THE SMALL INTESTINE

The experiments described in this thesis are concerned with the induction and expression of CMI responses in the small intestine. In this section I shall first review the properties of T lymphocytes and their role in the regulation of the immune response before turning to the features of mucosal T cells in particular. The latter part of this section will then deal with cell mediated immunity and its relationship to the intestinal mucosa.

T lymphocytes and regulation of the immune response

Although administration of antigen is normally associated with an active immune response directed at eliminating the antigen, it is clear that the consequences of antigen exposure fall within a spectrum of reactivity, ranging from unresponsiveness to active immunity. Immunological tolerance may be defined as the induction of a state of specific unresponsiveness by an antigen which under other circumstances is capable of inducing an immune response.

Specific tolerance may be induced by a wide range of antigens under many different experimental conditions with resulting unresponsiveness of all limbs of the immune response. The balance between tolerance and active immunity is delicate and is dependent on dose, nature and route of presentation of antigen and on the age and immune status of the animal. (Dresser & Mitchison 1968; Waksman 1977). Neonatal animals appear particularly susceptible to tolerance induction (Billingham, Brent & Medawar 1953)while unusually large or small amounts of antigen are tolerogenic (Dresser &

Mitchison 1968). The importance of antigenic structure in the development of tolerance is evidenced by the ability of deaggregated proteins to induce tolerance in contrast to the immunogenic properties of the aggregated protein (Chiller, Habicht & Weigle 1971; Parks & Weigle 1980a). Similarly, the complexing of antigen with isologous gamma globulin (Aldo-Benson & Borel 1974) or with syngeneic spleen cells (Cheung, Sherr, Heghinian, Benacerraf & Dorf, 1978) as non-immunogenic carrier moieties favours the induction of tolerance. Intravenous administration of an antigen is more likely than other routes of parenteral immunisation to induce tolerance (Dvorak, Billote, McCarthy & Flax 1965; Asherson & Ptak 1970; Kaufmann & Hahn 1979) while oral administration of antigen is also suitable for tolerance induction (Tomasi 1980). Studies of systemic tolerance have employed a wide range of experimental conditions and it is often difficult to extrapolate results from one model to another. It is apparent, however, that the induction of tolerance rather than active immunity, is favoured by large doses of soluble antigen given intravenously or orally.

The mechanisms responsible for the state of unresponsiveness may also differ in the various systems used. There is good evidence from the work of different groups that in individual models, tolerance is accompanied by functional deletion of B cells (Aldo-Benson & Borel 1974; Parks & Weigle 1980a,b), deficiency of helper T cell function (Parks & Weigle 1980b, Endres & Grey 1980b) and activation of suppressor T cells (Basten, Miller, Sprent & Cheers 1974;

Sy, Miller & Claman 1977). Significantly, suppressor T cells have been demonstrated by several groups studying tolerance of CMI responses in mice (Phanuphak, Moorhead & Claman 1974; Sy et al 1977; Kaufmann & Hahn 1979; Asherson & Zembala 1980). In several instances, however, the tolerant state in vivo does not correlate with studies of individual cellular function and several regulatory mechanisms may be functioning in the intact animal. In this respect, the presence or absence of suppressor T cells need not relate to the unresponsiveness of an animal (Parks & Weigle 1980a).

The study of immunological tolerance, has in recent years, led to the concept that the immune response of an animal is normally under a considerable degree of control. In particular much interest has focussed on the role of T lymphocytes in these regulatory pathways. Thus, the full development of the antibody response requires the cooperation of B cells and a class of helper T cells (Mitchell & Miller 1968) while a separate class of suppressor T cells exists to inhibit both humoral and cell mediated immunity (Pierce & Kapp 1976). These helper and suppressor T cells differ in surface phenotype and other characteristics. Thus, in mice, helper T cells are radioresistant, recognise antigen only in the presence of macrophages and bear the Lyt $1^{+}23^{-}$ phenotype (Erb & Feldmann 1975; Cantor, Shen & Boyse 1976; Anderson & Warner 1976). Suppressor T cells on the other hand, are sensitive to radiation, adult thymectomy, and cyclophosphamide, are thought to bind antigen directly and are most likely of the Lyt $1^{-}23^{+}$ phenotype (Feldmann & Kontiainen 1976; Cantor et al 1976; Anderson & Warner 1976;

Röllinghoff, Starzinski-Powitz, Pfizenmaier & Wagner 1977). It is now apparent therefore, that the immune response is governed by a complex interplay of lymphocyte classes each performing an unique function. Immunological circuits involving interactions between several precursor, inducer and amplifier T cells are thought to be of particular importance in the generation of suppressor T cells (Schwartz, Askenase & Gershon 1978; Cantor & Gershon 1979).

The final expression of cell mediated immunity is dependent on the outcome of the balance between active immunity and suppression following immunisation. Suppressor T cells appear to play a major role in regulating the induction and expression of CMI (Sy et al 1977; Röllinghoff et al 1977; Gill & Liew 1978; Lage-Stehr & Diamantstein 1978) while the existence of a helper cell for CMI remains unproven. At present there is good evidence that the induction of the T-dependent antibody response and delayed hypersensitivity follow separate pathways. The induction of helper and DTH effector T cells show marked differences in their dose response to the same antigen (Ramshaw, Bretscher & Parish 1976). However, T_H and T_{DTH} share the same surface phenotype (Cantor & Boyse 1975; Huber, Devinsky, Cantor & Gershon 1976; Cantor et al 1976) and the same MHC-restricted antigen recognition pathway (Miller, Vadas, Whitelaw and Gamble 1976; Cantor & Boyse 1975). It remains to be proven conclusively therefore that these are functions of T cells of separate origin rather than alternative functions of the same cell.

The immune response consists of an intricate network of cellular interactions and presentation of antigen is the trigger which commits the system to the production of humoral immunity, CMI or tolerance. While T lymphocytes have been shown to have a crucial role in this network, the final outcome of immunisation is dependent on the relationships between antigen, antigen-presenting cells and several classes of lymphocyte. It is apparent that there are several points in the pathway which provide routes by which the response may be modulated.

T lymphocytes in the small intestine

In the small intestine, lymphocytes are found both in the organised lymphoid tissues of the Peyers Patches and mesenteric lymph nodes and are also scattered throughout the gut wall. At birth, the Peyer's Patches and MLN are small, undeveloped structures which rapidly become populated by lymphoid cells in the first few days of life. At this time the majority of these lymphocytes are thymus-derived (Raff & Owen 1971; Chanana, Schaedeli, Hess & Cottier 1973; Barg & Draper 1975). Shortly after weaning these tissues acquire the characteristic appearance of secondary lymphoid tissues with well-developed primary follicles, germinal centres and thymus-dependent areas (Waksman 1973; Parrott 1976).

In the mucosa itself, large numbers of lymphocytes are found in the lamina propria and submucosa and there are also a significant number within the epithelial layer. (Figs. 2.1 & 2).

Intraepithelial lymphocytes form a heterogenous population of small to medium sized lymphocytes with a few large, lymphoblastoid cells. (Toner & Ferguson 1971; Collan 1972; Glaister 1973a; Ferguson 1973; Marsh 1975a). The number of IEL is substantially reduced in antigen-deprived small intestine (Ferguson & Parrott 1972a), in athymic or thymectomised mice (Fichtelius, Yunis & Good 1968; Ferguson & Parrott 1972a) and in thymectomised birds (Bäck 1970a). In addition, the majority of IEL react positively with reagents specific for T lymphocytes in the mouse (Guy-Grand, Griscelli & Vassalli 1974; 1978) and it is clear that the IEL represent a population which consists predominantly of T lymphocytes.

Origin of mucosal T lymphocytes

It has been known for several years that B lymphocytes from the Peyer's Patches, MLN or thoracic duct will migrate specifically to the lamina propria of the small intestine after transfer into syngeneic recipients while peripheral B cells will not. The GALT are especially rich in precursors of mucosal IgA plasma cells (Craig & Cebra 1971; Guy-Grand et al 1974; McWilliams, Lamm & Phillips-Quagliata 1974; Rudzik, Perey & Bienenstock 1974; Husband & Gowans 1978). More recently, evidence has accumulated that T lymphoblasts from the GALT will show similar specific gut-seeking behaviour while T lymphoblasts from peripheral sites do not (Guy-Grand et al 1974; 1978; Rose, Parrott & Bruce 1976). In contrast, the migration patterns of small, recirculating T lymphocytes are unclear. Gut-specific localisation of small intestinal T cells has been reported in the sheep (Cahill, Poskitt,

Frost and Trnka 1977), but this has not been confirmed in other species. Studies performed recently in mice have ascribed this phenomenon to contamination of migrating cells with T lymphoblasts (Freitas, Rose & Rocha 1980). Significantly, autoradiographic studies on the localisation of MLN and TDL T lymphoblasts or of thymocytes have identified migration of labelled cells into the gut epithelium (Goldschneider & McGregor 1968; Sprent 1976; Guy-Grand et al 1978). In addition, topical radiolabelling of mouse Peyer's Patches with $^{3}HTdR$ results in the appearance of labelled intraepithelial T cells during the GvHR and in normal animals (Guy-Grand et al 1978). These studies support the concept that the IEL are a population of T lymphocytes.

Radiolabelling studies in vivo confirm the evidence from these lymphocyte traffic experiments that the IEL are derived from a recently divided pool of cells (Darlington & Rogers 1966; Bäck 1970b; Lemmel & Fichtelius 1971; Marsh 1975b; Guy-Grand et al 1978). This has been further supported by the use of morphological criteria and electron microscopy (Marsh 1975a), although other workers have contested these findings (Glaister 1973a; Otto 1973). The IEL migrate into the epithelium from the lamina propria and after a short stay in that position return to the lamina propria (Meader & Landers 1967; Toner & Ferguson 1971; Glaister 1973a). Although the possibility has not been rigorously excluded, it is unlikely that the IEL are merely shed from the villus along with effete epithelial cells.

Functions of mucosal T cells

The observation that the numbers of IEL are depressed in antigen-deprived intestine would suggest that these cells are involved in an immune response to intestinally derived antigens (Ferguson & Parrott 1972a). Recently, attempts have been made in various species to investigate the functions of isolated preparations of small bowel mucosal lymphocytes in the hope that this might clarify the role of these cells in vivo. However, the isolation procedures are often technically difficult and conflicting results have emerged. Thus, it has been reported that guinea pig IEL respond to PHA, Con A and pokeweed mitogen, while chicken IEL do not (Arnaud-Battandier,Wahl & Nelson 1979; Arnaud-Battandier, Lawrence & Bloese 1980). In addition, guinea pig IEL have been shown to exhibit spontaneous, mitogen-induced and antibody-dependent cell mediated cytotoxicity in vitro in a manner similar to spleen cells (Arnaud-Battandier,Bundy, O'Neill, Bienenstock & Nelson 1978). Several studies employing preparations said to consist of lamina propria lymphoid cells have identified lymphocytes which respond to PHA and Con A and also in the mixed lymphocyte reaction, in rabbits, humans and guinea pigs (Singal, O'Neill, Clancy & Bienenstock 1976; Arnaud-Battandier et al 1978, 1979; Goodacre, Davidson, Singal & Bienenstock 1979). Antibody dependent cell-mediated cytotoxicity has not been found in lamina propria lymphocytes of the small intestine, however (Arnaud-Battandier et al 1978). Although many of these findings require confirmation, it is apparent that the

epithelium and lamina propria of the small intestine contain lymphocytes which have the characteristic functions of mature T lymphocytes. This is supported by the findings that organ cultures of small intestine may produce migration inhibition factors for guinea pig macrophages (Gadol, Waldman & Clem 1976) and human leucocytes (Ferguson, McDonald, McClure & Holden 1975) although the cells producing these factors have not been identified.

Mucosal mast cells

The majority of IEL are lymphoid cells by morphological criteria and several workers have described a significant proportion of IEL which contains granules of similar staining characteristics to those of the mast cell series, although the granules of IEL are smaller than those of true mucosal mast cells (Murray, Miller & Jarrett 1968; Collan 1972; Rudzik & Bienenstock 1974). True mast cells are found rarely in the epithelium and in larger numbers in the lamina propria of the gut, particularly round the crypts. Mucosal mast cells differ from connective tissue mast cells in their morphological appearance (Enerbäck 1966a) and histochemical properties (Enerbäck 1966b). More interesting are the findings that they may be a thymus dependent population (Ruitenberg & Elgersma 1976) and that they are expanded during parasitic infestations (Miller & Jarrett 1971; Ruitenberg & Elgersma 1976) and during the GvHR (Guy-Grand et al 1978). While these last workers have identified T cell surface markers on mucosal mast cells and there is evidence of thymus dependency of these cells, the exact nature of the

relationship between mucosal T cells and mast cells is unknown.

Oral immunisation and tolerance

When an animal's first encounter with antigen is by the oral route, it responds with the production of local secretory antibodies of the IgA class (Dolezel & Bienenstock 1971a; Heremans 1974). In addition, small amounts of antigen are absorbed intact and may induce systemic humoral and cell mediated immunity (Uhlenhuth 1900; Dolezel & Bienenstock 1971b; Perrotto, Hang, Isselbacher & Warren 1974). However, oral administration of antigen is also extremely effective in inducing systemic tolerance to a subsequent parenteral challenge with the antigen. The phenomenon was first noted by Dakin (1829) who described the practice among American Indians of ingesting extracts of poison ivy to alleviate the symptoms which occurred on a subsequent, cutaneous encounter. Since then, systemic tolerance has been described after feeding proteins (Wells & Osborne 1911; Thomas & Parrott 1974; Vaz, Maia, Hanson & Lynch 1977; Ngan & Kind 1978), contact-sensitising agents (Chase 1946; Asherson, Zembala, Perera, Mayhew & Thomas 1977) and heterologous red cells (André, Heremans, Vaerman & Cambiaso 1975; Kagnoff 1978a,b). Furthermore, all effector limbs of the immune response may be rendered unresponsive in this way, including total serum antibody responses (Hanson, Vaz, Maia, Hornbrook, Lynch & Roy 1977; Kagnoff 1978a; Mattingly & Waksman 1978), IgE responses (Vaz et al 1977, Ngan & Kind 1978) and CMI responses (Chase 1946; Asherson et al 1977; Kagnoff 1978b).

The factors responsible for the systemic unresponsiveness are not yet clear and serum antibody (Kagnoff 1978a), antigen-antibody complexes (André et al 1975) and suppressor lymphocytes (Thomas & Parrott 1974; Asherson et al 1977; Ngan & Kind 1978; Mattingly & Waksman 1978) have all been implicated. In addition, the liver may have an important role in this phenomenon (Thomas & Parrott 1974). Irrespective of the exact nature of the regulatory mechanism, the induction of tolerance is apparently an important consequence of antigen feeding. This is of practical importance, since one of the objectives of my work was to study active intestinal immunity to a dietary antigen. In order to do so, I recognised that it might first be necessary to interfere with the regulatory functions which normally inhibit induction of active intestinal immune responses.

Cell mediated immunity

Immunisation of an animal with antigen may produce an immune response involving both humoral and cell-mediated immunity. Cell-mediated responses in vivo characteristically develop slowly after immunisation and the effector cells involved include macrophages and specific thymus-derived lymphocytes. In contrast, the humoral limb of the immune response responds quickly to antigen by the production of specific antibodies. In this section I shall deal with the characteristics of effector T cells, in contrast to the regulatory cells discussed earlier.

In the cellular response, the interaction of T lymphocytes with antigen results in the induction of delayed type hypersensitivity or the generation of cytotoxic effector cells

which destroy specific target cells. In recent years the nature of effector T cells has been the focus of much interest, and it has become clear that these cells bear surface phenotypes which are specific to their function. Thus, in the mouse, T cells responsible for the phenomenon of cell mediated lympholysis are of the Lyt $1^{-}23^{+}$ or Lyt $1^{+}23^{+}$ phenotype (Cantor & Boyse 1975; Beverley, Woody, Dunkley, Feldmann & McKenzie 1976) while T cells capable of transferring DTH and of cooperating with B cells in the antibody response are Lyt $1^{+}23^{-}$ (Vadas, Miller, McKenzie, Chism, Shen, Boyse, Gamble & Whitelaw 1976; Huber et al 1976; Cantor et al 1976). It is apparent that T lymphocytes respond to antigen in the context of cell-surface structures encoded by the major histocompatibility complex (Katz & Benacerraf 1975; Snell 1978) and it has been shown that mouse cytotoxic T cells react to antigens which are linked to the K and/or D regions of the H-2 locus. T_{DTH} on the other hand are restricted by antigens of the I region (Cantor & Boyse 1975; Blanden, Doherty, Dunlop, Gardner & Zinkernagel 1975; Miller et al 1976; Wolters & Benner 1979). These findings indicate that while cytotoxicity and DTH are both products of effector T cell activation, these phenomena reflect the actions of two subclasses of T lymphocyte which are quite distinct in nature and function. Although both forms of immunity may be induced during a CMI response, it is important to recognise that either pathway, or both, may be the predominant feature of any individual response.

In contrast to the target cell killing which results from direct cell-cell contact and is mediated by cytotoxic T cells, the effector mechanisms involved in the action of T_{DTH} reflect the release of soluble mediators by activated lymphocytes (lymphokines). In this way, a CMI response is amplified by the action of lymphokines on other lymphocytes (T and B) and on many accessory cells including macrophages, polymorphonuclear leucocytes and eosinophils. While it has been shown that antibodies to lymphokines can inhibit delayed skin lesions in the guinea pig (Yoshida, Bigazzi & Cohen 1975), the in vivo role of soluble mediators in the pathogenesis of DTH is unknown. Similarly, the significance of circulating lymphokines in human disease is unclear (Cohen, David, Feldmann, Glade, Mayer et al 1977). The production of lymphokines by sensitised lymphocytes in vitro, however, has been invaluable in the measurement of CMI responses. Of these, the production of mediators which inhibit the migration of macrophages and polymorphs in vitro correlates well with the presence of systemic DTH in vivo (David, Al-Askari, Lawrence & Thomas 1964a,b; Bloom & Bennett 1966; Bloom 1971) and provides a simple in vitro assay for its assessment. (Table 2.1).

In recent years, the cells responsible for lymphokine production have been identified and these studies have reinforced the relation between lymphokine production and the effector limb of DTH. Thus, lymphokines are produced in vitro during the thymus-dependent response to mitogens (Namba, Jegasothy & Waksman 1977), protein antigens (Bloom &

Bennett 1966) and alloantigens (Kasakura & Lowenstein 1965) and are therefore consistently associated with immune responses involving T-cell activation. Furthermore, while B cells may secrete lymphokines in response to mitogenic stimulation, antigen-induced production of mediators is dependent on the presence of T cells (Rocklin, McDermott, Chess, Schlossman & David 1974; Rasanen 1979). More recently, Lyt 1^+ T cells have been found to be responsible for lymphokine production in mice (Kuhner, Cantor & David 1980), in agreement with the subclass responsible for DTH in vivo.

In addition to the T lymphocytes and macrophages normally associated with DTH responses in vivo, recent work has suggested a role for additional accessory cells in the pathogenesis of these reactions. Thus, it has been proposed that the full expression of DTH reactions in the skin requires the participation of mediators released by cells of the basophil and mast cell series (Gershon, Askenase & Gershon 1975). The precise function of such mediators in DTH responses is unknown, but it is clear that the induction and expression of delayed type hypersensitivity requires complex interactions between committed T cells and several types of accessory cell.

Cell mediated immune responses in the small intestine

The large numbers of T-cells present in the gut mucosa (and GALT) would imply the existence of active CMI reactions occurring locally in the small intestine. The following two main roles for T lymphocytes in the gut mucosa are likely:

firstly local CMI may serve to protect the gut against pathogenic organisms and alternatively, CMI responses to environmental antigens (e.g. food proteins) may be responsible for intestinal damage in susceptible individuals. Evidence that such responses do occur has come from the following systems.

a) Allograft rejection of gut: Transplantation of allografts of jejunum in dogs has been shown to produce villous atrophy, crypt hyperplasia and lymphocytic infiltration of the mucosa and subsequent invasion of the graft mucosa by bacteria (Holmes, Klein, Winawer & Fortner 1971). Since bacterial infection may have been responsible for this damage, rejection of "antigen-free" allografts of foetal gut implanted under the kidney capsule of adult animals was studied subsequently. In these investigations, rejection of gut occurred within 10-20 days of transplantation and was a thymus-dependent phenomenon (Ferguson & Parrott 1973; MacDonald 1976). Although villus atrophy did occur, this was a late feature and was preceded by enhanced crypt mitotic activity, crypt lengthening and lymphocytic infiltration of the mucosa and epithelium (Ferguson & Parrott 1973; MacDonald & Ferguson 1976; 1977). The role of CMI in this phenomenon was underlined by the absence of polymorphs and plasma cells in the lesions (MacDonald & Ferguson 1976) and by the fact that rejection preceded the anti-allograft antibody response (Elves & Ferguson 1975).

b) Graft-versus-host reaction: In 1954, Barnes and Loutit reported that lethally irradiated mice could not be reconstituted with allogeneic spleen tissue. Subsequently, Billingham and Brent (1957) described a runting syndrome in a proportion of neonatal mice given allogeneic lymphocytes. Graft-versus-host reaction is now recognised to occur in animals receiving allogeneic lymphocytes to which they are unresponsive and has been shown to be due to the presence of small, alloreactive T cells in the inoculum (Cantor & Asofsky 1972; Kerckhaert, Benner & Willers 1973).

Diarrhoea and wasting are prominent features of the GvHR in experimental animals (Reilly & Kirsner 1965; Cornelius 1970) and contribute significantly to the morbidity and mortality of patients developing GvHD following bone marrow transplantation (Slavin & Santos 1973; Glucksberg, Storb, Fefer, Buckner, Neiman, Clift, Lerner & Thomas 1974). In the small intestine of runting animals the lesions consist of villous atrophy with crypt hyperplasia and in severe cases, degeneration and necrosis of the mucosa may occur (Reilly & Kirsner 1965; Cornelius 1970; Wall, Rosenberg & Reilly 1971; Elson, Reilly & Rosenberg 1977). This intestinal damage has been shown to produce mucosal enzyme deficiencies (Hedberg, Reiser & Reilly 1968), malabsorption (Palmer & Reilly 1971) and protein losing enteropathy (Cornelius 1970) in affected animals. Similar pathological changes and malabsorption have been observed in patients with GvHD (Slavin & Santos 1973; Glucksberg et al 1974; Woodruff, Hansen, Good, Santos & Slavin 1976). In addition, examination

of the intestinal mucosa of these patients and of mice with GvHR has revealed infiltration with lymphocytes and other mononuclear cells (Slavin & Santos 1973; Woodruff et al 1976; Guy-Grand et al 1978).

During the GvHR, there is a large expansion of cytotoxic cells (Cerottini, Nordin & Brunner 1971; Singh, Sabbadini & Sehon 1972) and cell-mediated cytotoxicity is the conventional explanation for tissue damage during the GvHR. However, damage may occur during the GvHR in skin (Billingham & Streilen 1968; Streilen 1972) and kidney (Elkins & Guttman 1968) even if the tissues are syngeneic to the injected alloreactive cells. This "Innocent Bystander" phenomenon has also been recently described in grafts of foetal small intestine using conventional histology as the measure of intestinal injury (Elson et al 1977). Further evidence that the mucosal lesions of the GvHR are not due to direct cytotoxicity to epithelial elements comes from a study which identified a stimulation of crypt mitotic activity as the initial response during the GvHR (MacDonald & Ferguson 1977). The exact relationship between mucosal changes and the GvHR remains to be clarified.

c) Parasitic infections of the small intestine: Cell mediated immunity has been implicated in both the defence of the intestine and in the mucosal damage during parasite infections. Thus, the villous atrophy and crypt hyperplasia occurring in rats infected with Nippostrongylus brasiliensis and in mice infected with Trichinella spiralis has been shown to be a thymus-dependent phenomenon (Ferguson & Jarrett 1975;

Manson-Smith, Bruce & Parrott 1979). Furthermore, in the latter model, the mucosal alterations are preceded by an influx of T lymphoblasts to the intestinal wall (Manson-Smith, Bruce, Rose & Parrott 1979) and T lymphocytes are necessary for the transfer of adoptive immunity to this parasite (Wakelin & Wilson 1979). Similar studies on murine giardiasis have also indicated that T cell mediated immunity is responsible for protection against the parasite (Roberts-Thomson & Mitchell 1978; Stevens, Frank & Mahmoud 1978) and for the resulting crypt hyperplasia (MacDonald & Ferguson 1978). In addition, increased IEL counts are found in mice and humans with giardiasis (MacDonald & Ferguson 1978; Ferguson, McClure & Townley 1976). Lastly, the expansion of mucosal mast cells during N. brasiliensis infection is transferrable by immune sIg^- thoracic duct lymphocytes (Nawa & Miller 1979) and is thymus-dependent during infection with T.spiralis (Ruitenberg & Elgersma 1976).

d) Local cell mediated immune responses to fed antigen: While it is reported that systemic CMI may result from oral administration of antigen (Perrotto, Hang, Isselbacher & Warren 1974), such studies may be complicated by the risk of systemic immunisation, and there is little known of the induction of CMI in the mucosa itself. Prolonged feeding of a contact-sensitising agent, DNCB, to mini-pigs was shown to produce small intestinal injury including villous necrosis, polymorph and mononuclear cell infiltration and xylose malabsorption (Bicks, Azar & Rosenberg 1967). However, although true contact sensitivity following topical application

of contact agents to the colon is well documented (Bicks, Azar, Rosenberg, Dunham & Luther 1967; Askenase, Boone & Binder 1978) this phenomenon has not been confirmed in the small intestine. Contact-sensitising agents are themselves toxic to the mucosa and systemic unresponsiveness has been shown to be the more usual immunological consequence of feeding these substances (Chase 1946; Glaister 1973b; Asherson et al 1977).

Several workers have however, reported that oral immunisation may induce local production of MIF in the small intestine. Thus, MIF was shown to be produced by isolated mucosal lymphoid cells and MLN lymphocytes from pigs fed DNP-BGG (Huntley, Newby & Bourne 1979), while in discussion, Bienenstock has reported the production of MIF by mucosal and Peyer's Patch lymphocytes from BCG-fed guinea pigs (O'Neill & Bienenstock 1977). Similarly, migration inhibition factors are produced by the small intestine of guinea pigs fed cholera organisms (Gadol et al 1976) and by mucosal lymphocytes from pigs infected orally with transmissible gastroenteritis virus (Frederick & Bohl 1976). In none of these instances however, have the studies identified the exact source of the factors produced, nor have alterations in mucosal structure or function been reported. Furthermore, as previously discussed, systemic tolerance is the usual result of feeding non-viable antigens.

e) Cell-mediated immunity in small intestinal disease:

Coeliac disease involves the development of villous atrophy and crypt hyperplasia in the small intestinal mucosa,

and probably is due to an immune response to the cereal protein, gluten (Lancet 1974). Evidence that this reflects a CMI response comes from the observations of increased numbers of IEL in coeliac disease (Ferguson 1974; Mavromichalis, Brueton, McNeish & Anderson 1976;) and reports of the presence of CMI in peripheral lymphocytes of affected patients (Bullen & Losowsky 1978). Furthermore, small intestinal biopsies from untreated patients produce leucocyte inhibition factor when challenged with gluten in vitro (Ferguson, MacDonald, McClure & Holden 1975).

In childhood, severe degrees of villous atrophy and crypt lengthening may result from an intolerance to cow's milk protein (Visakorpi & Immonen 1967). While it is not yet clear what the exact nature of the hypersensitivity reaction is, these children have increases in IEL count (Phillips, Rice, France & Walker-Smith 1979; Rosekrans, Maijer, Cornelisse, Wal & Lindeman 1980) and crypt mitotic activity (Kosnai, Kuitunen, Savilhati, Rapol & Kőhegyi 1980) similar to those in coeliac disease.

Measurement of CMI responses in the mucosa and intestinal lymphoid tissues

Since intestinal antibody responses are dissociated from systemic humoral immunity (Dolezel & Bienenstock 1971a,b) it is likely that assays for systemic CMI will not reflect the presence of such immunity in the gut itself. This is supported by the observations that local, pulmonary CMI does not correlate with systemic CMI following inhalation of antigen (Henney & Waldman 1970; Yamamoto, Anacker & Ribi 1970;

Clancy & Bienenstock 1974). However, the relative inaccessibility of the small intestine renders conventional in vivo assays of CMI impractical while isolation of mucosal lymphoid cells is not yet sufficiently established to allow routine in vitro measurement of CMI in the mucosa itself. It is probable, however, that the gut associated lymphoid tissues will provide suitable organs for such studies.

Since the MLN is a rich source of gut-seeking T lymphoblasts (Guy-Grand et al 1974, 1978; Rose et al 1976) it is likely that the intestinal immune response develops in the MLN and migrates subsequently to the gut wall and immunological events in the MLN may therefore reflect immune responses in the mucosa itself. CMI responses in lymphoid tissues may be assayed in vitro by lymphocyte transformation or by production of lymphokines in the presence of antigen. Both assays require varying periods of in vitro culture and, as in the case of lymphokine production, may necessitate a two-step culture method. While most assays employing migration inhibition as an in vitro correlate of CMI now use phagocytic cells as indicators, the first descriptions of these techniques employed entire explants of lymphoid tissues (Rich & Lewis 1932; Carpenter, Barsales & Ganghan 1968). Subsequently, it was reported that whole lymphoid cell populations taken from the lymphoid organs of immunised rabbits showed migration inhibition in the presence of the appropriate antigen and that this correlated with systemic DTH (Likhite, Haasz, Algom & Richter 1972). A more recent study describing a similar phenomenon in thymocytes from

immunised mice (Hughes, Preece & Light 1980) suggests that a migration inhibition assay of this type might be applicable to the GALT and the MLN in particular. This would provide a simple in vitro assay for intestinal CMI responses.

The existence of "enteropathic lymphokines" has been proposed to explain the features of CMI responses occurring in the intestinal mucosa (Ferguson & MacDonald 1977). In addition, lymphokine production by intestinal mucosa has been described in experimental animals (Frederick & Bohl 1976; Gadol et al 1976) in humans with coeliac disease (Ferguson et al 1975) and in rejected allografts of foetal gut (Ferguson & MacDonald 1977). These findings indicate that specific production of lymphokines by mucosal lymphocytes may prove a useful assay for local intestinal CMI. However, the source and function of such mediators is unknown and their identification in large numbers of specimens would be technically unsatisfactory. Furthermore, the difficulties in isolating mucosal lymphocytes precludes the exploitation of mucosal lymphokines as a routine assay for mucosal CMI at present.

However, it is likely that alternative, if indirect, parameters of mucosal CMI may exist within the mucosal architecture itself. In the models of intestinal CMI discussed earlier, consistent increases in crypt cell production rate and crypt depth were demonstrated. In addition, these were shown to be early and prolonged responses of the mucosa in both allograft rejection and

GvHR (MacDonald 1976; MacDonald & Ferguson 1977). These observations indicate that alterations in crypt cell kinetics may reflect mucosal CMI. In addition, the T cell nature of intraepithelial lymphocytes and the fact that their numbers are increased during allograft rejection of gut (MacDonald 1976; MacDonald & Ferguson 1976) support the concept that the IEL may be an additional index of intestinal CMI. These parameters should be equally applicable in CMI responses to both soluble antigens and cellular antigens.

Factors acting on T and B lymphocytes.

1) Helper
   - Mitogenic factor
   - T cell replacing factor
   - Allogeneic effect factor
   - T cell growth factor

2) Suppressor
   - Soluble immune response suppressor
   - Inhibitor of DNA synthesis
   - Histamine-induced suppressor factor
   - Antibody inhibitory material
   - Lymphoblastogenesis inhibitory factor

Factors acting on accessory cells.

- Macrophage migration inhibition factor
- Macrophage activating factor
- Leucocyte migration inhibition factor/leucocyte migration enhancement factor
- Chemotactic factors for: neutrophils, eosinophils, basophils, monocytes
- Eosinophil stimulation promoter
- Granulocyte phagocytosis enhancement factor
- Platelet aggregating factor
- Osteoclast activating factor(s)
- Fibroblast mitogenic factor
- Collagen synthesis enhancing factor (fibroblasts)

Cytostatic/cytotoxic factors.

- Proliferation inhibitory factor
- Lymphotoxin

Others.

- Type II Interferon
- Skin reactive factor
- Angiogenesis factor
- Lymph Node permeability factor.

Table 2.1. Antigen non-specific factors released by activated lymphocytes (lymphokines).

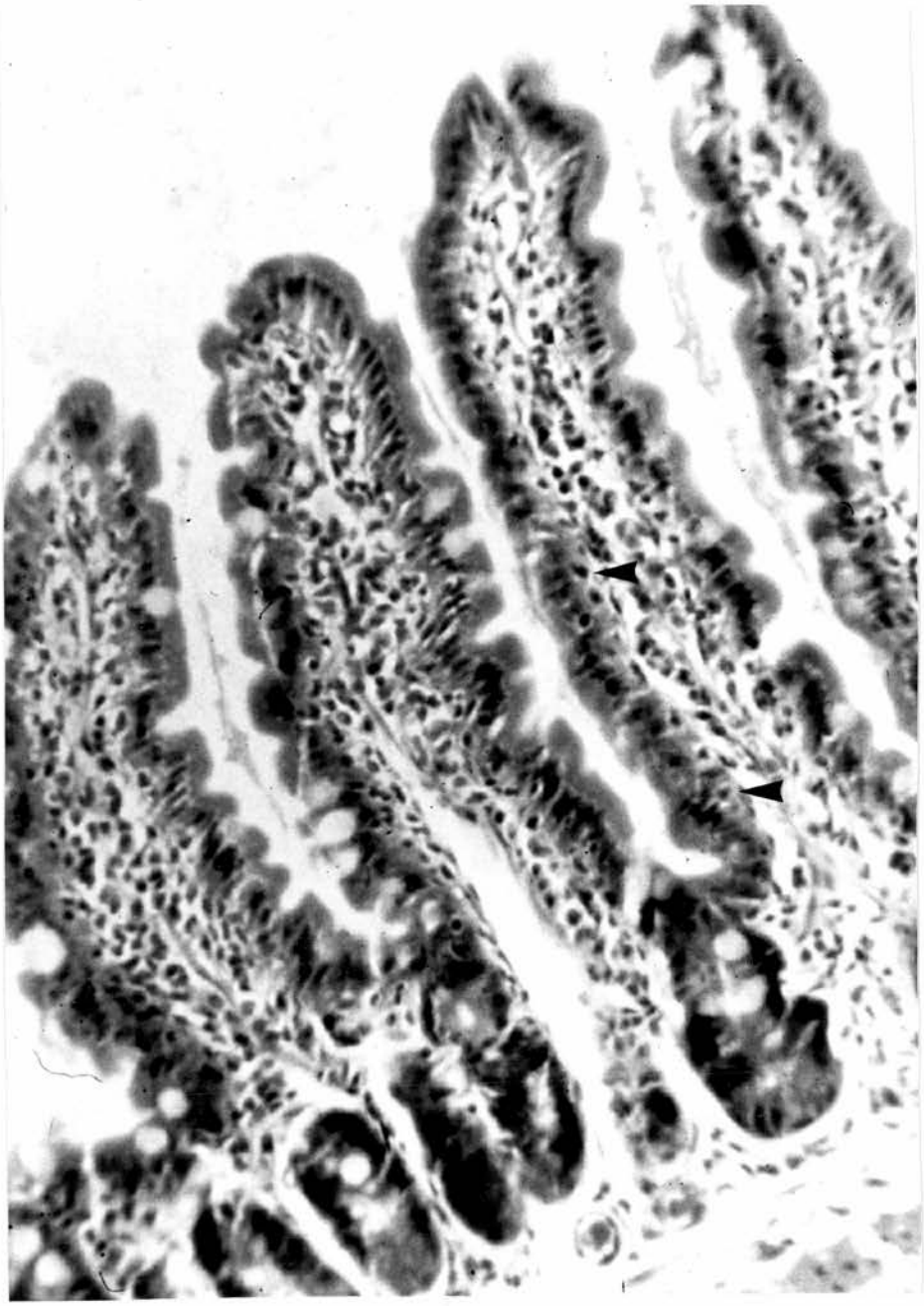


Fig. 2.1. Histology of the normal mouse small intestine showing intraepithelial lymphocytes (arrows). (H & E x 500)

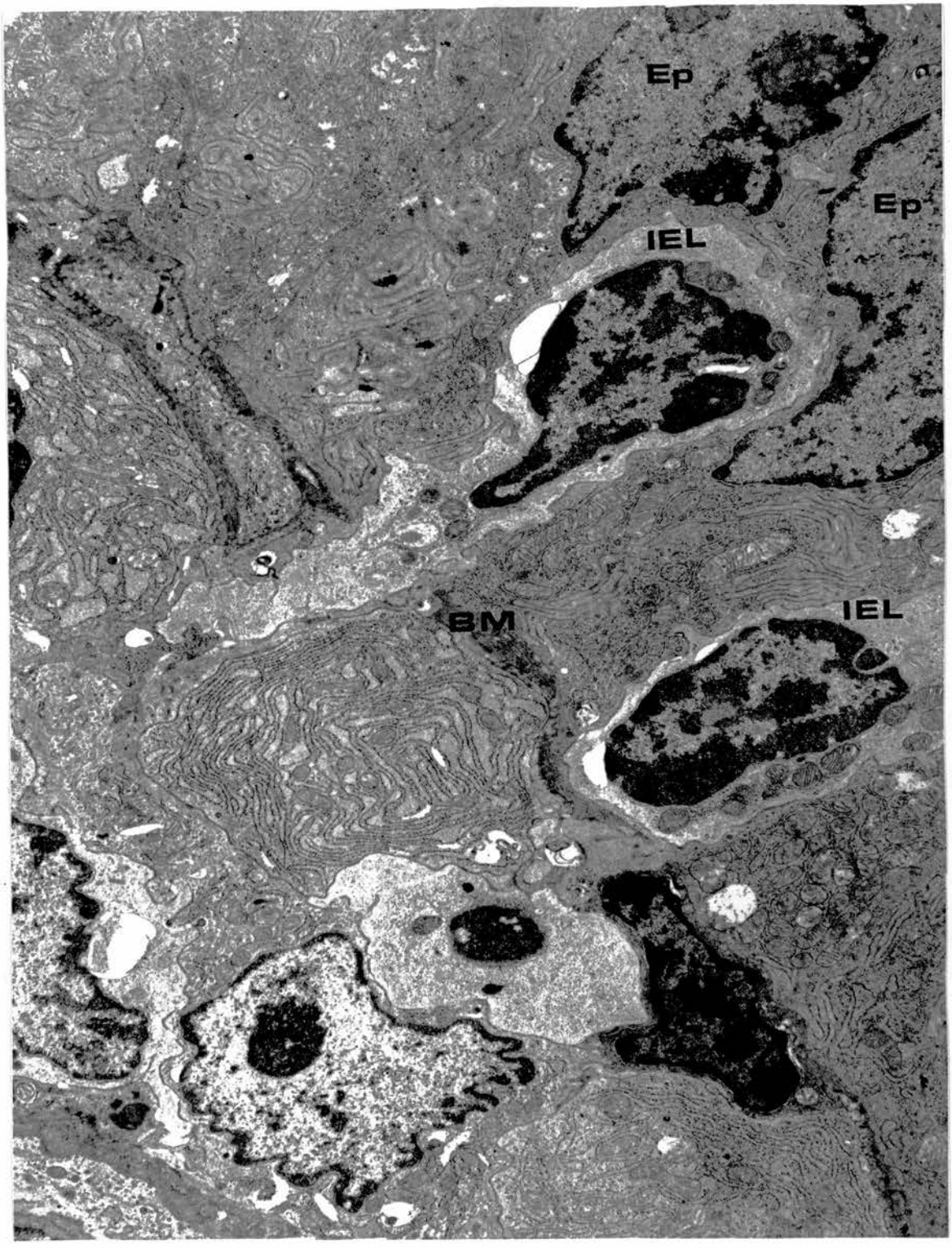


Fig. 2.2. Two intraepithelial lymphocytes in mouse jejunum (IEL). Note tail of IEL which has crossed basement membrane (B.M.) (x 16,000) (Ep - epithelial cell nucleus).

CHAPTER 3

EPITHELIAL CELL KINETICS IN THE SMALL INTESTINE

In many of the experiments described in this thesis, the crypt cell production rate was chosen as one of the principal indices of the effector limb of mucosal CMI. It is therefore necessary to consider the epithelial cell kinetics of the intestine and to discuss briefly the regulation of crypt cell turnover.

The surface of the small intestinal mucosa consists of a single layer of columnar epithelial cells which forms a continuous sheet from the base of the crypt to the tip of the villus. In addition to the functional absorptive cells of the epithelium (enterocytes) this layer also contains lymphocytes, mucus-producing goblet cells and endocrine cells.

The architecture of the mucosa is based on the villus/crypt unit in which cell loss from the villus tip is normally balanced by the production of new cells in the crypts (Fig. 3.1). Epithelial cells are formed by proliferation of slowly dividing stem cells at the base of the crypt which thereafter migrate upwards onto the surface of the villus (Wright 1980). During this period terminal differentiation into functional enterocytes occurs in the maturation zone near the mouth of the crypts and the cells continue to mature as they move up the villus (Cairnie, Lamerton & Steel 1965; Imondi, Balis & Lipkin 1969). In mice, the enterocytes remain on the villus for 2-3 days before being shed from the mucosa into the gut lumen (Leblond & Messier 1958).

Control of cell renewal in the mucosa

In the steady state of the normal small intestine there is a delicate balance between cell loss and production, the outcome of which determines the final architecture and function of the mucosa. The crypt mitotic activity is subject to a wide range of regulatory mechanisms which allow cell production to adapt rapidly to environmental changes. These control functions have been well reviewed recently and include systemic and local humoral factors, neurogenic control, dietary changes and local bacterial flora (Eastwood 1977; Williamson 1978a,b). In addition, the size of the functional compartment of the villus may have an important role in influencing cell production in the crypts. Thus, depletion of villous cell numbers by ionising radiation or ischaemia provokes a reactive increase in crypt cell production rate which returns to normal when the villous compartment attains its normal size (Rijke, van der Meer-Fieggen & Galjaard 1974; Rijke & Gart 1979).
In addition, a similar mechanism has been proposed to explain the increased cell turnover in the crypts of patients with coeliac disease (Watson & Wright 1974). While crypt hyperplasia may frequently reflect an adaptation to villous damage, it is also likely to occur as a result of imbalance in the regulatory factors normally controlling crypt cell turnover. In this respect, several humoral factors directly influence crypt cell kinetics, while increased cell turnover has been shown to occur in the absence of villous damage after administration of cytostatic drugs (Roti Roti & Dethlefsen 1975).

In addition to the epithelial elements of the mucosa, the entire villus/crypt unit is closely invested with a mesh of supporting fibres throughout the ground substance of the mucosa. While the exact nature of the relationship is not clear, it has been proposed that the ultimate morphology of the mucosa is dependent on the function of mucosal fibroblasts which maintain this ground tissue (Parker, Barnes & Kaye 1974; Marsh & Trier 1974). Furthermore, abnormalities of the supporting structures have been described in the presence of altered cell kinetics in patients with coeliac disease (Loehry & Creamer 1969; Asquith, Johnson & Cooke 1970).

Measurement of crypt cell proliferation kinetics

Some years ago, a simple and reliable method of assessing the mitotic activity of rat small intestinal crypts which avoided the need for radioisotope technology was described (Wimber & Lamerton 1963). The technique is based on the ability of colchicine to block all dividing cells at metaphase, allowing metaphases to accumulate for several hours after administration of the drug. If animals are killed at intervals after colchicine and a significant linear relationship can be established between metaphase accumulation and time, the rate of cell production/crypt/hour can then be derived from the slope obtained by regression analysis. Although this method only assesses cell production/crypt and cannot estimate the total cell output per villus, it has proved a reliable means of estimating crypt cell proliferation kinetics under many

conditions. In addition to those investigating mucosal CMI responses (MacDonald 1976; MacDonald & Ferguson 1977) other workers have employed the technique to monitor epithelial cell kinetics in both experimental animals and in human disease (reviewed by Wright & Appleton 1980).

In addition, the technique involves fixation of tissue without shrinkage and the specimens can be used for accurate measurement of villous and crypt size. Previous work has shown this to be more reliable than quantifying these parameters on histological sections (Clarke 1970) and these can then be estimated in parallel with assessment of crypt cell mitotic activity.

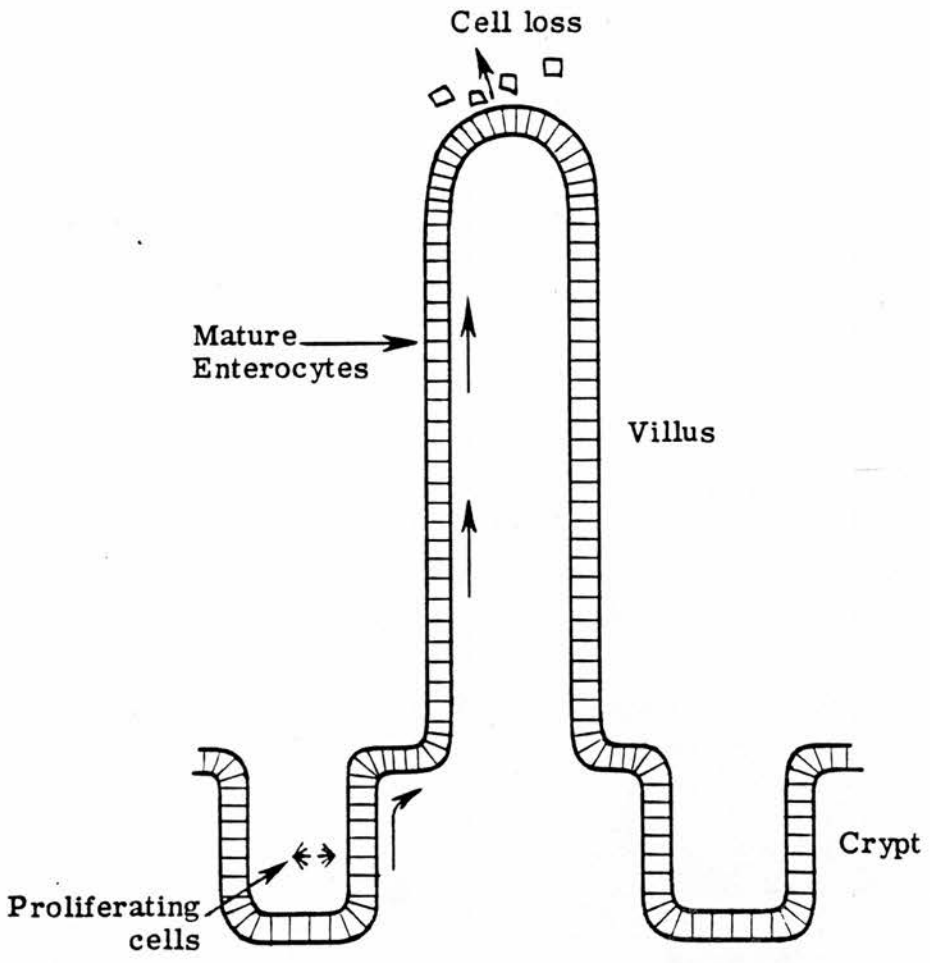
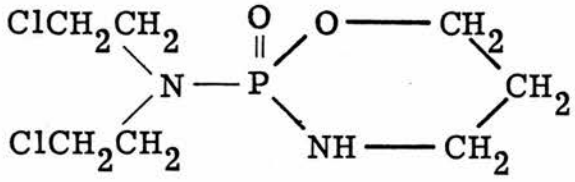


Fig. 3.1. The villus/crypt unit of the normal small intestine. Cell loss from the villus tip is normally balanced by replacement of cells in the crypts.

CHAPTER 4

CYCLOPHOSPHAMIDE

Cyclophosphamide (2 [Bis(2-chloroethyl)amino] tetrahydro-2H-1,3,2-oxazaphosphorine 2-oxide) is a potent alkylating agent used as a chemotherapeutic agent in the treatment of malignant and autoimmune disease.



After parenteral administration, the drug is metabolised by microsomal enzymes in the liver (Foley, Friedman & Drolet 1961; Connors, Cox, Farmer, Foster & Jarman 1974) active metabolites are released and are transported to target cells via the bloodstream. In mice, CY has a serum half-life of around 45 minutes and it has completely disappeared from serum by 2-3 hours after i.p. injection (Mellett 1967). Since it acts principally on cells actively synthesising DNA its toxic effects are on tissues with a large proportion of dividing cells, including tumour tissue, bone marrow and the epithelia of the urinary and gastrointestinal tracts. In addition, it has been proposed that highly susceptible tissues are deficient in a CY-detoxifying enzyme (van Putten & Lelieveld 1970; Connors et al 1974).

Cyclophosphamide and immunity

The effects of CY on the immune system were first detailed by Turk and Poulter (1972) who demonstrated that in the dose range 300-400 mg/kg, CY depleted mainly the B cell areas of the spleen and lymph nodes of guinea pigs and mice. Since the thymic cortex was also depleted by the

drug and bone marrow reconstitution accelerated recovery, it was proposed that CY principally affected short-lived B and T cells. The longer-lived, recirculating T cell pool was relatively spared. This work has been confirmed by several groups and it has been shown that maximal depletion of B cell areas occurs within 2-3 days of a single dose, with a regenerative phase beginning a few days thereafter. During regeneration, the lymphoid tissues become populated by large, dividing cells and the normal architecture is lost (Turk & Poulter 1972; Stockman, Heim, South & Trentin 1973; Kolb, Poupon, Lespinats, Sabolovic & Loisillier 1977). In those studies in which effects on Peyer's Patches are reported a similar pattern and time course of events has been observed (Chin & Hudson 1970; Mazigh, Alonso & Mollaret 1979).

When CY is given to animals shortly after immunisation, variable effects on the immune response are reported, but the predominant result is a dose-related suppression of immunity which mainly affects antibody synthesis (Mitsuoka, Baba & Morikawa 1976; Kerckhaert, Hofhuis & Willers 1977; Ramshaw, Bretscher & Parish 1977). These results reflect the ability of CY to prevent cellular proliferation during an immune response. However, if CY is given 2-3 days before immunisation, a different pattern emerges. In this case, the antibody response may be normal or reduced, but depending on the dose used, cell mediated immunity may be enhanced. In the mouse, selective enhancement of CMI has been shown

to occur in doses between 20 and 200 mg/kg, with larger doses producing progressive depletion of CMI (Lagrange, MacKaness & Miller 1974; Askenase, Hayden & Gershon 1975; Kerckhaert et al 1977; Easmon & Glynn 1977; Gill & Liew 1978). At first, the concomitant depletion of B cell areas and antibody responses led authors to suggest that the increased CMI responses were due to the loss of blocking effects of antibody (Turk, Parker & Poulter 1972; Lagrange et al 1974). In recent years however, it has become increasingly apparent that many of the immunological effects of the drug are explicable by its inhibitory action on suppressor T cells, and it has been shown that the suppressor cell precursors are exquisitely sensitive to CY treatment. Given before antigen, CY prevents the generation of TS from short-lived precursors but does not affect the mature effector T lymphocytes responsible for DTH and cytotoxicity (Rollinghoff et al 1977; Schwartz et al 1978; L'age-Stehr & Diamantstein 1978; Attallah, Ahmed & Sell 1979). These in vivo effects of CY have been elegantly confirmed by recent in vitro studies which show that TS and B cells were more sensitive to active metabolites of CY than helper and effector T cells (Kaufman, Hahn & Diamantstein 1980; Shand & Liew 1980). In the guinea pig, enhancement of contact sensitivity by CY has been found to be secondary to elimination of suppressor B cells (Asherson et al 1977). In contrast to the large number of studies of lymphocyte function, the effects of CY on accessory cells have received

little direct attention. However, it has been shown by one group that CY has little effect on phagocytosis by macrophages and only slightly inhibits intracellular killing by these cells (Sharbaugh & Grogan 1969; Sharbaugh 1976). Others have reported macrophage activation by functional and morphological criteria after repeated CY treatment (Buhles & Shifrine 1978). Relative sparing of these cells is further indicated by the rapid recovery of blood monocyte counts and macrophage stem cells following CY (Hunninghake & Fauci 1976; Buhles and Shifrine 1978).

Cyclophosphamide and the small intestine

Since CY acts on rapidly dividing tissues, the epithelial layer of the small intestine provides an obvious target organ. Administration of CY in a range of doses has been shown to produce a dose-dependent inhibition of crypt mitotic activity within 6 hours of dosing, reaching a maximum by 24 hours. Thereafter, a large increase in mitotic activity occurs and above normal levels of crypt cell proliferation are seen until 3 days after treatment (Rosenoff, Bostick & Young 1975; Ecknauer 1976; Young 1976; Sobhon, Wanichanon & Sretarugsa 1977). The resulting effects on the intestinal epithelium include necrosis and extrusion of crypt cells which is maximal within 24 hours (Ecknauer & Löhrs 1976; Sobhon et al 1977). Levels of epithelial cell enzymes may fall at later times and this is thought to reflect immature epithelial cells reaching the villus during the period of rapid cell turnover (Hartwich, Weisshaar & Domschke 1978).

In the gut CY affects principally the dividing crypt cell population, and even large, sublethal doses of the drug do not appear to injure mature enterocytes (Waldeck 1972). This observation is confirmed in rats where it has been shown that a single dose of 100 mg/kg had minimal direct effects on either enzyme activity or sugar absorption (Ecknauer 1976; Ecknauer & Löhrs 1976; Hartwich et al 1978). Detailed studies on the functional capacity of the CY-treated gut in vivo are lacking and there is no information on the influence of CY on handling of protein antigens by the small intestine.

CHAPTER 5

MATERIALS AND METHODS

Animals:

Mice used were of the following inbred strains: CBA/Ca ($H\text{-}2^{k/k}$ Mls^{b}), C3H/He ($H\text{-}2^{k/k}$ Mls^{c}), and BALB/c ($H\text{-}2^{d/d}$). (CBAxBALB/c) F_1($H\text{-}2^{k/d}$) and (CBAxC3H) F_1($H\text{-}2^{k/k}$ $Mls^{b/c}$) hybrids were bred from CBA mothers and C3H or BALB/c fathers. NZW rabbits were used to raise anti-ovalbumin antisera and mixed strain Guinea pigs as a source of complement. All animals were bred and maintained in the Animal Unit, Western General Hospital, Edinburgh. Adult mice were normally used at 6-10 weeks of age.

Diet:

Animals were normally maintained on a standard rodent diet (Spratts Rodent Diet No.1) and had access to tap water ad libitum.

In some experiments, groups of mice were fed 2 or 20 mg/100 ml Ovalbumin (Sigma Fraction V) incorporated in their drinking water, equivalent to a daily dose of 0.1 or 1 mg/mouse.

Anaesthesia:

Procedures such as footpad injection were carried out under light ether anaesthesia. Surgical operations were performed using Sagatal 60 mg/ml (M & B Ltd.) diluted 1:10 in sterile water. Mice were injected i.p. with 0.1 ml/10 g body weight and this provided adequate anaesthesia in most cases for up to 2 hours. Where necessary this was supplemented by light ether anaesthesia.

Sacrifice of animals:

Mice were killed by cervical dislocation.

Bleeding of mice:

Small quantities of blood were obtained routinely from the retro-orbital plexus under ether anaesthesia using heparinised haematocrit tubes (Propper Ltd). In this way, approximately 200 µl could be obtained from each mouse at regular intervals. When larger quantities of blood were required, mice were bled out from the axillary vein.

Removal of tissues:

Organs, especially jejunum, were obtained for histology immediately after sacrifice. Pieces of jejunum (5 mm x 5 mm) were removed 10 cm from the pylorus and avoiding obvious lymphoid aggregates. These were opened and placed villous surface upwards on pieces of card for fixation. Organs such as spleen, thymus and liver were sliced and one section placed in fixative. Intestinal grafts were removed from the kidney capsule intact, and whenever possible opened out with fine, pointed scissors.

Body weights:

Mice were weighed using an Oertling TP40 single pan balance.

Organ weights:

Organs were dissected free of surrounding tissues and weighed on a torsion balance (White Electrical Instrument Co.).

Histology:

After removal of tissues they were immediately placed in fixative. For conventional histology, jejunum, spleen, Peyers Patches and liver were fixed in 10% phosphate buffered formalin, while thymus and mesenteric lymph nodes were fixed

in Bouins' Fluid. These were embedded in paraffin wax and sections 4 μm thick cut. Intestinal grafts were serially sectioned along the entire length of the graft after embedding.

Tissues processed in this way were stained with Haematoxylin and Eosin.

Jejunal specimens were also taken for mast cell staining. In this case, pieces of jejunum were removed as before and fixed in Carnoy's Solution. After processing, these were stained with Astra Blue - Safranin pH 0.3.

Histological specimens were examined using a Leitz Ortholux II microscope.

Histological processing was carried out by Mr F. Donnelly and Mr A. Sutherland.

Intraepithelial lymphocyte counts:

IEL were counted by the method of Ferguson and Murray (1971) and are expressed as the number of IEL/100 epithelial cells. Specimens were examined under x 400 magnification and only sections with a single cell layer were counted. Differential cell counts were performed by counting epithelial and lymphoid cell nuclei lying above the basement membrane and a total of 600 epithelial cells counted in each specimen. In sections of heterotopic grafts it was occasionally impossible to count 600 cells and in these cases, at least 200 epithelial cells were counted. Care was taken to avoid recurring areas in serial sections of these grafts.

Mast cell counts:

Mucosal mast cells occurred predominantly in the lamina propria round the crypts and at the base of the villi. Occasional non-epithelial cells with positively staining granules were seen in the epithelial layer and these were included in the count. The cells were counted using a square-grid eyepiece fitted to the Leitz Ortholux microscope calibrated to give the number of cells/mm^2. The eyepiece was orientated with the lower edge lying on the upper surface of the muscularis mucosae and in areas where the grid was almost filled with mucosa. Tissues were examined at x 400 magnification, and in each specimen, five well orientated areas were counted and the mean number of mucosal mast cells expressed /mm^2 mucosa.

Processing of tissues for microdissection:

The method of Clarke (1970) was employed. Mice were injected with 7.5 mg/kg colchicine (BDH Ltd.) i.p. and sacrificed at intervals of 20-120 min. thereafter. Pieces of jejunum were removed and fixed in Clarke's Fixative for 6-24 hours and then stored in 75% Ethanol before use. Tissues were stained in bulk by the modified Feulgen reaction. The pieces of gut were immersed in 50% Ethanol for 10 minutes, followed by 10 minutes in tap water and 7 minutes in 0.1 N HCl at 60°C. After a further 10 minutes in tap water, the specimens were flushed x 3 with fresh tap water, stained with Schiff reagent (Difco Ltd) for 30-40 minutes at room temperature and kept in tap water for microdissection. Under the dissecting microscope (x 32 Zeiss Stereomicroscope 4B)

the muscularis mucosae was first stripped off. Thin slices of mucosa each containing a few villi and their surrounding crypts were then cut from the edge by dissection with a cataract knife (Weiss Ltd.) and forceps. The fragments were then placed on a microscope slide in 45% acetic acid, covered with a coverslip and examined under the Leitz microscope with a previously calibrated eyepiece micrometer. In each specimen, the lengths of 10 villi and 10 crypts were measured and the means taken. The pieces were then gently squashed under the coverslip and the number of metaphases per crypt counted. Once again the mean of values for 10 crypts was used.

The crypt cell production rate was obtained in the following manner: for each time interval after colchicine, the mean number of metaphases/crypt is taken and the pairs of results plotted by linear regression analysis. Having established linearity, the CCPR is calculated from the gradient of the line of best fit calculated by the method of least squares. Fig. 5.1 shows the results from a typical experiment.

Assessment of lymphoid follicles in heterotopic grafts of small intestine:

Sections obtained by serial sectioning of grafts were orientated in identical fashion on the slide and stained with HeE. Individual lymphoid follicles were easily identified and those appearing in different positions in the graft circumference were counted as separate follicles.

Assay of epithelial cell enzymes:

5 mm^2 pieces of jejunum were removed, gently cleaned of adherent debris and weighed before storage at -20°C. The brush border enzymes lactase and sucrase were assayed by the method of Dalqvist (1964) and the results expressed as mmol of substrate hydrolysed/minute/g wet weight of tissue at 37°C. Disaccharidase estimations were performed by Dr D. Al Thamery.

Preparation of cell suspensions:

Spleens, thymus, mesenteric and peripheral lymph nodes were removed immediately after sacrifice. After washing in RPMI 1640 (Flow Labs) they were dissected free of surrounding material and sliced with scissors. The pieces were then gently passed through a fine gauge wire mesh using the plunger of a 5 ml syringe (BD Ltd) and the resultant suspension passed once through a 23 g needle to break up clumps. The cells were allowed to stand for a few minutes at room temperature to allow debris to settle out and washed x 3 at 400 g in supplemented RPMI 1640. After counting in a heamocytometer (Neubauer) the final cell pellet was made up to the required concentration in complete RPMI 1640 for use. Cell viability was assessed by exclusion of 2% eosin and was normally greater than 90%.

Purification of T cells by nylon wool columns:

The method of Julius et al (1973) was followed. 0.6 g nylon wool which had previously been washed in distilled H_2O for 1 week at 37°C was packed loosely into a 10 ml syringe

and the column washed through with complete RPMI 1640 supplemented with 5% foetal calf serum (Flow Labs). The column was drained and incubated at 37°C for 1 hour before use. Lymphoid cell suspensions in RPMI 1640 + 5% FCS were passed over a glass wool column to remove debris and dead cells and after washing in fresh medium, were allowed to pass into the nylon wool column which was then incubated for 45 minutes at 37°C. Non-adherent cells were then flushed out with warm RPMI 1640 + FCS, washed once and resuspended for use. This was found to produce populations of 97% T cells from lymph nodes as assessed by fluorescent staining for the Thy 1.2 marker.

Elimination of T cells from cell suspensions:

2×10^7 lymphoid cells/ml of complete RPMI 1640 were incubated for 30 minutes at room temperature in 1/1000 dilution of monoclonal IgM anti-Thy 1.2 antiserum (F7D5 - Olac 1976). The cells were then washed x 3 in medium, resuspended in a 1/10 dilution of fresh guinea pig serum as a source of complement and incubated at 37°C for 40 minutes. Cells were washed 3 times before use. In cytotoxicity assays, killing was assessed by eosin exclusion.

Identification of lymphocyte classes by immunofluorescence:

To identify sIg^+ cells approximately 10^7 lymphoid cells in phosphate buffed saline pH 7.2 were incubated in a few drops of FITC-labelled goat anti-mouse Fab_2 (Gibco Ltd), for 45 mins at 4°C and washed x 3 in PBS. Cells were examined for positive fluorescence using a Wild M20 microscope fitted with dark-ground condenser and HBO-200 mercury vapour lamp

with exciting filter for light of 495 nm wavelength and barrier filter for light of 525 nm wavelength.

Thy 1.2-bearing cells were identified indirectly. Approximately 10^7 lymphoid cells in PBS were suspended in a few drops of a 1:200 dilution of monoclonal anti-Thy 1.2 for 45 minutes at room temperature, washed x 3 in PBS and resuspended in FITC-labelled goat anti-mouse Fab_2. Further steps were as above. The proportion of Thy 1.2 positive cells was determined as follows:

% Thy 1.2 positive = % staining after (anti-Thy 1.2 + anti-mouse Fab_2) - % staining with anti-mouse Fab_2 alone.

Induction of Graft-versus-host reaction:

Adult CBA mice were used as sources of donor spleen cells in all cases. Recipients were either (CBA x BALB/c) F_1 or (CBA x C3H) F_1 adults or neonatal (CBA x BALB/c) F_1 mice. Adult mice received 6 x 10^7 CBA spleen cells in 0.2 ml intra-peritoneally while neonatal animals received 2.5 x 10^7 CBA cells in 0.1 ml RPMI 1640 i.p. 5-7 days after birth. Control mice received either equivalent numbers of F_1 cells or medium alone. In the case of neonatal mice, care was taken to ensure that littermate controls were used for each individual experiment.

Graft-versus-host reaction assay:

The Spleen Weight assay of Simonsen was used (Simonsen 1962). On the day of sacrifice, mice were weighed, the spleen removed and weighed, and the relative spleen weight expressed as mg/10 g body weight.

The Spleen Index is given as

$$\frac{\text{Mean Relative Spleen Weight in Mice with GvHR}}{\text{Mean Relative Spleen Weight in Control Mice.}}$$

In addition, differences between mean relative spleen weights of each group were compared by Student's t-test.

Implantation of foetal grafts of intestine under the kidney capsule:

This technique has been described in detail previously (Ferguson & Parrott 1972; Ferguson 1973) and is briefly as follows: Foetuses were removed from pregnant mice on the 18th or 19th day of gestation, the small intestine removed and 5-10 mm segments cut and placed in ice-cold saline. The kidneys of anaesthetised adult mice were exposed through lateral incisions in the abdomen and the kidney capsule incised with a scalpel blade (Gillette Scimitar). One segment of foetal gut was placed under each capsule using a plastic cannula (Argyle Medicut) and the peritoneum and skin closed. Grafts were allowed to grow for 4 weeks before GvHR was induced in the host animals.

Cyclophosphamide:

Mice were given 100 mg/kg cyclophosphamide (Endoxana WB Ltd) in saline intraperitoneally.

Antigens:

Five times recrystallised fractions of ovalbumin, human serum albumin and bovine serum albumin (Sigma Ltd) were used to immunise experimental animals. In addition, oxazolone (4 ethoxymethylene-2-phenyl-oxazol-5-one BDH Ltd) was dissolved in olive oil at 37°C for oral immunisation.

In practice, however, this proved unsatisfactory for routine oral administration, since it was impossible to obtain accurate concentrations of oxazolone in solution.

Parenteral immunisation of animals:

Mice were immunised for studies of systemic immunity with either 2 mg antigen in Complete Freund's Adjuvant (H37Ra-Difco Ltd) i.p. or 100 µg antigen in CFA intradermally to one rear footpad. In studies of local immunity, mice were immunised with either 100 µg or 10 µg in OVA in CFA distributed between three or four footpads, and the draining lymph nodes subsequently removed. In one experiment, mice were immunised with 100 µg OVA in Incomplete Freund's Adjuvant (Difco Ltd) distributed between all footpads.

Rabbits were immunised with 2 mg OVA in CFA intramusculary at monthly intervals for 3 months and bled out 2 weeks after the last injection.

Oral immunisation of mice:

Unanaesthetised mice were fed OVA in distilled water intraoesophageally using a rigid, steel feeding tube with a rounded end.

Passive haemagglutination assay:

Sera were tested for the presence of haemagglutinating anti-OVA Ab by passive haemagglutination. Sheep red blood cells in Alsever's solution were washed three times in saline at room temperature, and 200 µl packed cells mixed with 1.4 ml saline and 200 µl protein solution (15 mg/ml OVA or

1 mg/ml HSA in saline). 2 ml of 0.01% chromic chloride (Analar BDH Ltd) in saline at pH 5 was then added dropwise with continuous agitation and the mixture allowed to stand at room temperature for 10 min. After stopping the reaction by addition of phosphate buffered saline
the coated cells were then washed twice in PBS and finally resuspended at 1%.

Sera to be tested were inactivated at 56°C for 30 min. and absorbed with 10% SRBC before use. 25 µl serum was doubly diluted in round-bottomed microtitre plates (Titertek Ltd) and 25 µl coated SRBC added to each well. The plates were allowed to settle for 90 min. at room temperature and the titre taken as the last dilution to show complete agglutination. All sera were tested with or without the presence of 25 µl 0.1M 2-mercaptoethanol (Sigma Ltd) to obtain levels of both IgM and IgG class antibodies. Haemagglutination assays were performed in collaboration with Mrs H. Drummond.

Assays for systemic delayed-type hypersensitivity:

Mice were tested for DTH by one of two intradermal skin test methods. In the first instance, mice were shaved on both flanks, and the double skinfold thickness measured before and 24 hours after 100 µg Ag in 0.05 ml saline intradermally using a pair of skin calipers (Pocotest A - Carobronze Ltd). Other mice were tested by the increase in footpad thickness 24 hours after 100 µg Ag in 0.05 ml saline intradermally, measured in a similar fashion. In each case, immunised mice were also tested with 0.05 ml saline as controls.

Detection of sensitisation in local lymphoid tissues by lymphocyte migration inhibition:

Mesenteric lymph nodes were removed from orally immunised animals while the popliteal, axillary and brachial lymph nodes were taken from animals immunised in all footpads with antigen in CFA. After processing, cell suspensions were adjusted to 1.5 x 10^8/ml in complete RPMI 1640 and 10 µl capillary tubes (Drummond Microcaps) filled with the cells. After sealing one end with paraffin wax (Cristaseal-Hawksley Ltd), these were pelleted at 400 g for 5 mins. and the tubes cut just above the cell pellet. The parts containing the cells were then placed in wells of migration inhibition plates (Sterilin Ltd), being held in place with silicone vacuum grease (Edwards Ltd). The wells were then filled with 0.5 ml complete RPMI 1640 alone or medium containing various concentrations of Ag, sealed with a coverslip and incubated for 21 hours at 37°C. After culture, the areas of cell migration were drawn using a camera lucida (Zeiss Ltd) attached to a Zeiss stereomicroscope and measured by planimetry. The results are expressed as a Migration Index obtained as follows:

$$\text{M.I.} = \frac{\text{Area of migration in wells containing antigen}}{\text{Area of migration in medium alone}}$$

A minimum of 6 wells was used for each Ag concentration.

Morphological characteristics of migrating cells:

After overnight incubation and measurement of migration areas, the coverslips were removed from the wells, and the capillary tubes discarded. The migrated cells remaining in

the wells were then removed by Pasteur pipette, and aliquots spun on a cytocentrifuge (Shandon Cytospin) at 1000 rpm for 5 mins. The resulting preparations were dried in air, stained with Giemsa (Gurr Ltd) for 10 minutes and mounted in D.P.X (BDH Ltd) for examination.

In addition, cells were pelletted at 400 g, resuspended in fresh RPMI 1640 and adherent cells allowed to settle on glass coverslips at 37°C for 4 hours in 5% CO_2 in air. The coverslips were then gently washed in PBS, fixed in methanol and stained with Giemsa as before.

Detection of circulating antigen in ovalbumin-fed mice:

To detect circulating OVA a passive haemagglutination inhibition assay was employed. Serial dilutions of 25 µl test serum or normal mouse serum containing 2 mg/ml OVA were made in microtitre plates (Titertek Ltd) and 25 µl 1/1000 rabbit anti-OVA added to each well. After incubation for 30 minutes at room temperature, 25 µl OVA-coated SRBC (prepared as above) were added, and the wells allowed to settle. The titre of the test sera was taken as the last well to show complete absence of haemagglutination, and the OVA concentration calculated from the titre of a standard OVA containing serum.

Assay for the detection of ovalbumin in mouse diet:

20 g of dry mouse pellets were ground up to a fine power before stirring overnight in 100 ml phosphate-buffered saline pH 7.2 at 4°C. The resulting suspension was spun at 15,000 g for 30 minutes, the supernatant removed

and a small aliquot stored at 4°C for use after filter sterilisation (Millipore 0.22 μm). The remaining supernatant was adjusted to the isoelectric point of OVA, pH 4.6 and saturated ammonium sulphate pH 4.6 added dropwise to a final concentration of 60% ammonium sulphate. The precipitate obtained was centrifuged at 15,000 g for 30 minutes, washed in fresh 60% ammonium sulphate pH 4.6 and resuspended in 20 ml PBS at pH 7.2. The solution was extensively dialysed against distilled water, pH 7.0, to remove remaining salts, and a final volume of 25 ml obtained. A crude estimate of protein content was obtained by measuring the OD_{280nm} on a UV spectrophotometer (Pye Unicam SP 800A) previously calibrated with dilutions of ovalbumin in PBS. Both the crude supernatant and the ammonium sulphate fraction were assayed for the presence of OVA by the haemagglutination inhibition assay previously described.

Processing of tissues for transmission E.M.:

1 mm^2 pieces of tissue were fixed immediately in 5% glutaraldehyde in 0.1 M sodium cacodylate buffer pH 7.3 for 2-24 hours. Tissues were embedded in fresh araldite (Agar Aids Ltd), polymerised at 60°C for 3 days and 60 nm sections cut on an ultramicrotome (LKB 111). After staining in saturated uranyl acetate and lead citrate the sections were viewed using a Phillips 300 electron microscope.

All tissue processing for TEM was performed by Mr A. Sutherland.

Processing of tissues for scanning E.M.:

Pieces of jejunum, 1-2 cm in length, containing a Peyer's Patch, were fixed in 2.5% gluteraldehyde in cacodylate buffer pH 7.3 for at least 24 hours at $4^{o}C$ after pinning out, villous surface upwards, on a cork base. After washing for one hour in fresh cacodylate buffer and then distilled water, tissues were taken to 100% acetone and subjected to critical point drying in CO_2 using acetone as the transition medium. They were then mounted on aluminium stubs, coated with gold in a sputter coater and examined on an ISI 60 Scanning Electron Microscope.

SEM studies were performed in collaboration with Miss F. Allan.

Statistics:

Results are expressed as means $\pm$ 1 standard deviation, unless otherwise stated. Student's t-test was used to compare differences between groups in most cases. In experiments including haemagglutination assays, non-parametric distributions were observed and results were compared by Wilcoxon's Rank Sum test in addition to the Student t-test. In practice, similar significance levels were usually obtained.

Crypt cell production rates, calculated by linear regression were compared by covariance analysis to detect differences between the slopes. All calculations were performed on a Texas T1-5I-111 calculator.

Solutions:

Phosphate Buffered Saline pH 7.2 (5 litre)

NaCl 36.0 g

Anhydrous Na_2HPO_4 7.49g

Anhydrous KH_2PO_4 2.15g

Complete RPMI 1640 Medium

RPMI 1640 (Flow Labs) 100 ml

Penicillin (5000 u/ml)/Streptomycin (5000 µg/ml) (Flow Labs) 2 ml

Hepes Buffer (Flow Labs) 1M 1.5 ml

Glutamine (Flow Labs) 0.2M 2 ml

10% Buffered Formalin - 1 litre

Formaldehyde 100 ml

Distilled Water 900 ml

$NaH_2PO_4.2H_2O$ 4 g

Na_2HPO_4 6.5 g

Clarke's Fixative - 1 litre

Ethanol 750 ml

Glacial Acetic Acid 250 ml

Bouin's Fluid - 1.050 litre

Saturated aqueous picric acid (2,4,6-trinitrophenol) - 750 ml

Formaldehyde 250 ml

Glacial Acetic Acid 50 ml

Carnoy's Fixative - 1 litre

Ethanol 600 ml

Chloroform 300 ml

Glacial acetic acid 100 ml.

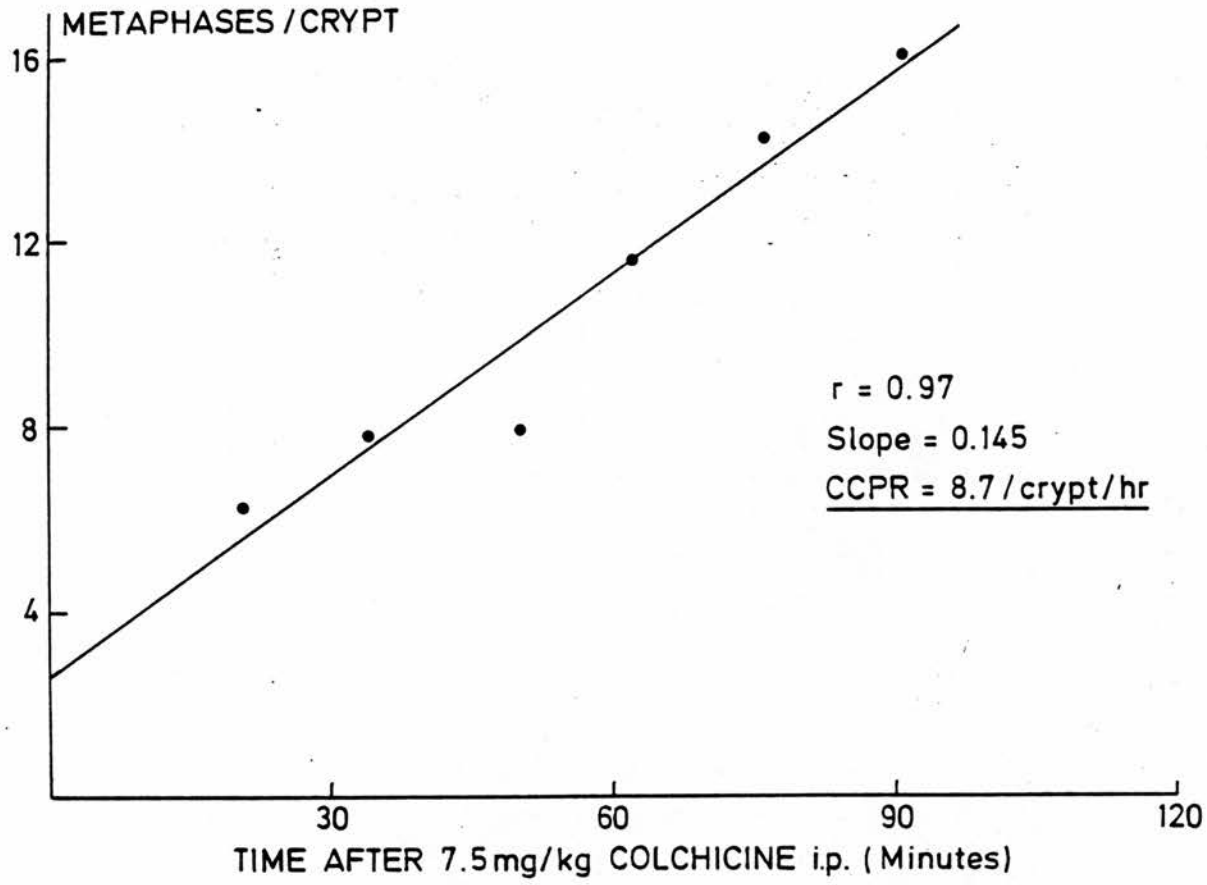


Fig. 5.1. Metaphase accumulation in normal mouse jejunum following injection of cokhicine. Each point represents mean number of metaphases in 10 crypts from one mouse. Animals were killed at various intervals after cokhicine and slope is calculated by method of least squares.

CHAPTER 6

EVOLUTION OF MUCOSAL CHANGES DURING THE GRAFT-VERSUS-HOST REACTION IN NEONATAL F_1 MICE

Introduction

Shortly after the induction of a GvHR in mice, a state of delayed hypersensitivity to host histocompatibility antigens develops in the animal (Wolters & Benner 1978). In addition, small intestinal damage is one of the principal pathological consequences of GvHR in experimental animals (Reilly & Kirsner 1965; Cornelius 1970; Hedberg et al 1968). This association indicates that the intestinal phase of the GvHR provides an important model of mucosal CMI in the small intestine.

The earliest response of the mucosa during a GvHR has been shown to be a stimulation of crypt cell mitotic activity with a subsequent increase in crypt length (MacDonald & Ferguson 1977). Similar changes in CCPR during allograft rejection of gut are associated with a rise in IEL count (MacDonald & Ferguson 1976; 1977) and lymphocytic infiltration of the gut mucosa is a feature of the GvHR in mice (Guy-Grand et al 1978) and humans (Woodruff et al 1976). These observations suggest that enhanced crypt cell production and lymphocytic infiltration are predictable consequences of a GvHR occurring in the intestine. However, earlier workers have not related these features to other, systemic indices of the GvHR and have not followed the mucosal changes for longer than 14 days after induction of the GvHR. I have therefore tested the hypothesis that the CCPR and IEL count provide reliable parameters of mucosal CMI by correlating changes in mucosal architecture and IEL count with the progress of the GvHR over a prolonged period, using the Spleen Index

(Simonsen 1962) as a measure of the proliferative phase of the GvHR. Irradiation itself damages the small intestine and I wished to assess only the effects of the CMI involved in the GvHR on the mucosa. Thus, the mice used as hosts in these experiments were unirradiated F_1 neonates injected with parental spleen cells for induction of the GvHR.

Increased numbers of mucosal mast cells and granulated lymphocytes have also been described in the intestinal phase of the GvHR in mice (Guy-Grand et al 1978). Since these cells are reported to be thymus-dependent (Ruitenberg & Elgersma 1976), the relationship of mucosal mast cells to the other features of the GvHR in the intestine has also been investigated in these experiments.

Experimental design

Male and female (CBA x BALB/c)F_1 mice were injected with 2.5×10^7 CBA spleen cells i.p. at 5-7 days of age and sacrificed at intervals from 1-62 days thereafter. The mice were weighed, the Spleen Index calculated and pieces of jejunum processed for histological examination and micro-dissection as described previously. At all times, a group of littermate controls which had received F_1 spleen cells or medium alone were also sacrificed.

Development of the GvHR

As assessed by the Spleen Index, significant splenic hypertrophy occurred from 3 days after transfer of parental spleen cells, reached a peak of 4.58 on day 11 and declined thereafter to a level of 1.65 which was maintained until the

end of the experiment (Fig. 6.1). This plateau of splenic hypertrophy most likely represents a continued non-specific hyperplasia of myeloid and erythroid elements in the spleen. The growth rate of neonates with GvHR was identical to their littermate controls, confirming that the increase in relative spleen weight was a reflection of splenic hypertrophy only. (Fig. 6.1)

Mucosal architecture during the GvHR

Subjective examination of histological sections revealed only crypt lengthening and mononuclear infiltration of the mucosa between 8 and 14 days after induction of the GvHR. (Figs. 6. & 6.3)

However, objective measurements made by microdissection showed significant alterations in mice with GvHR. Fig. 6.4 shows that the CCPR was markedly increased to 4.1/crypt/hour in these animals 3 days after induction and that a significant increase was found by 4 days (5.4 vs 1.6 $p < 0.005$). The upward trend reached a peak after 11 days (10.6 vs 1.4 $p < 0.001$), after which the CCPR declined, although values significantly greater than controls were maintained until day 18. Thereafter, the CCPR was consistently higher in mice with GvHR until normal values were reached on day 62. Associated with this increased crypt mitotic activity there was an accompanying increase in crypt length (Fig. 6.5). The crypt length of controls remained constant until day 8 of the experiment, after which the onset of weaning provoked a steady increase in crypt length. A similar pattern was seen in the CCPR of control mice. In mice with GvHR, there was already a significant increase in crypt length 2 days

after induction of GvHR (64.9 ± 1.7 mμ vs 59.5 ± 1.4 mμ $p < 0.02$). A steady rise to a maximum of 128.5 ± 8.3 mμ on day 8 (Controls 64.7 ± 1.7 mμ $p < 0.001$) was followed by a decline in crypt length from day 11. It should be noted however, that minor but significant increases in crypt length were observed until day 29 (106.2 ± 0.9 mμ vs 99.9 ± 3.2mμ $p < 0.01$). Normal values were found on day 62. In contrast to these crypt changes, villous shortening was not observed at any time during the GvHR (Fig. 6.6).

Cellular infiltration of the mucosa in GvHR

Intraepithelial lymphocytes were counted on H&E stained sections of jejunum, while mucosal mast cells were assessed on Astra Blue/Safranin stained sections fixed in Carnoys' solution.

Fig. 6.7 shows that there was an increased IEL count already 24 hours after induction of the GvHR (4.92 ± 0.79/100 epithelial cells vs 2.72 ± 0.35 $p < 0.01$). This result was rather surprising, and it was necessary to exclude random migration of injected spleen cells as a cause of a non-specific rise in IEL numbers. Injection of F_1 neonates with 2.5×10^7 F_1 spleen cells i.p. did not give a similar rise in IEL count 24 hours after transfer (1.96 ± 0.16 IEL/100 Ep cells) and it must therefore be assumed that the high IEL count after 1 day of the GvHR represents a true consequence of the response to F_1 tissues. After a small drop in IEL count on day 2, there was thereafter a steady rise in the IEL count to a maximum of 13.1 ± 2.14 compared to controls 2.8 ± 0.85

($p < 0.001$) on day 11. Significant increases in IEL count continued to be seen until day 18, despite a falling trend, but from then onwards, IEL numbers in GvHR and control mice were increasingly similar. On day 62, identical values were found in the two groups.

Mast cell numbers showed greater variability, particularly in younger animals where the small size of specimens made counting difficult. Thus no definite pattern can be observed in mast cell numbers until 6-8 days after induction of the GvHR (Fig. 6.8). However, after this time, there was a large expansion of mast cells reaching a peak at 14 days of 46.9 $6.8/mm^2$ compared to $8.5 \pm 8.0/mm^2$ for controls ($p < 0.001$). In contrast to the other indices investigated, mast cell numbers remained significantly increased until day 29 ($34.7 + 17.6/mm^2$ vs $9.8 \pm 4.8/mm^2$ $p < 0.05$). By 62 days however, both groups had identical values. It is also noteworthy, that in the villi of control animals, intra-epithelial lymphocytes were never found to stain positively for mast cell granules. In mice with GvHR however, occasional positively staining cells could be seen in the villous epithelium at x 400 magnification from day 14 onwards, while under oil immersion (x 1000), such cells were frequently identifiable in these sections. These stained IE cells did not resemble the more easily visible mast cells of the lamina propria. (Figs. 6.9 & 6.10).

Correlation of mucosal indices with progress of the GvHR

While assessment of the values on mice with GvHR gave clear patterns of development during the GvHR, it was essential

to correlate certain of the parameters with the Spleen Index, in order to assess their value as reproducible indices of mucosal CMI. Since most of the indices in control mice changed with age, particularly after weaning, the values in mice with GvHR have been expressed as a ratio to that of their littermate controls and these ratios compared with the Spleen Index. The ratios for indices of mucosal architecture compared with the Spleen Index are shown in Fig. 6.11 those relating to cellular infiltration in Fig. 6.12. It will be seen from these figures that the CCPR, crypt length and IEL count showed a close relationship to the Spleen Index throughout the experiment and the following correlations were obtained: CCPR vs Spleen Index $r = 0.74$ ($p < 0.005$), crypt length vs Spleen Index $r = 0.76$ ($p < 0.005$) and IEL vs Spleen Index $r = 0.86$ ($p < 0.001$). During the proliferative phase of the GvHR, up to day 11, the correlations between these features and the Spleen Index were even closer: CCPR $r = 0.88$ ($p < 0.005$); crypt length $r = 0.82$ ($p < 0.01$) and IEL $r = 0.99$ ($p < 0.001$). Finally, Fig.6.12 shows clearly the delayed mast cell response compared to the other parameters.

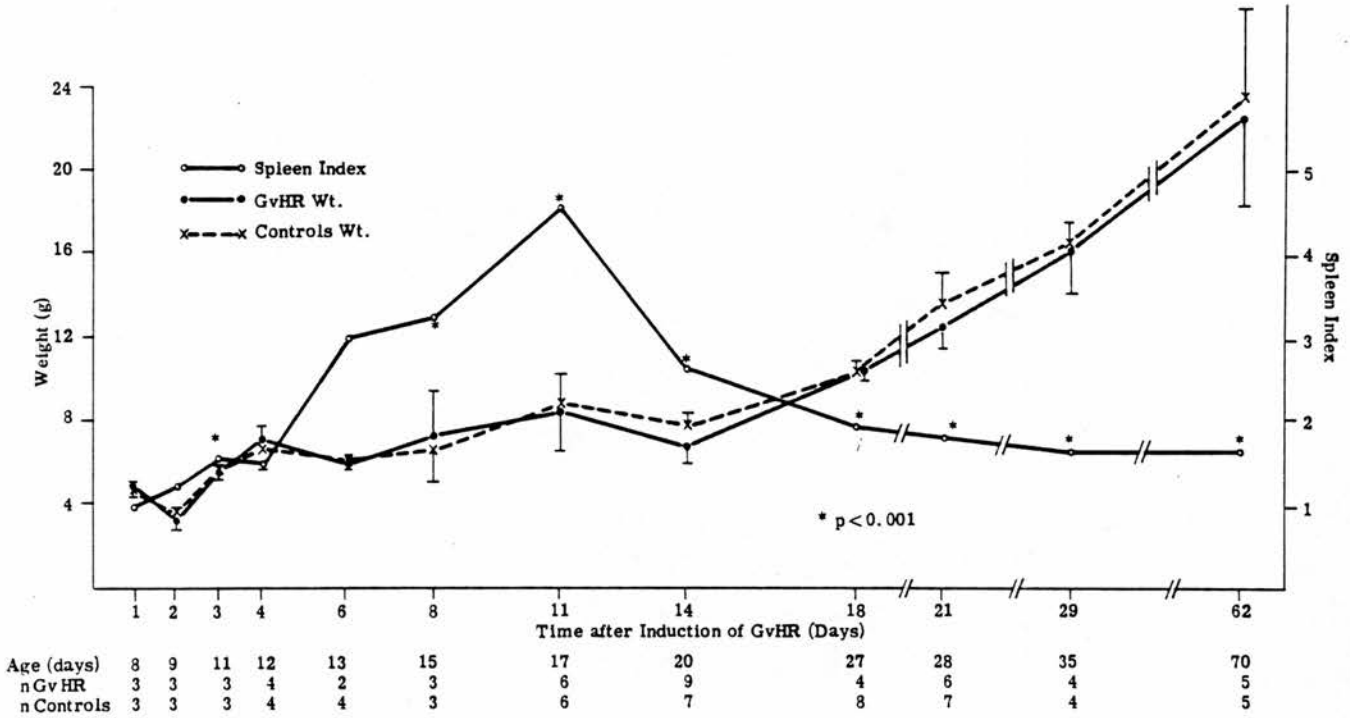
Conclusions

The results of these experiments have shown that when a Graft-versus-host reaction is induced in neonatal F_1 mice by i.p. injection of parental spleen cells, consistent and significant effects may be observed in the small intestinal mucosa. The principal of these are an expansion in crypt cell proliferation and crypt size, an increased number of lymphocytes in the epithelium and infiltration of the mucosa

by mast cells. The morphology of the villi are unaltered by this process.

Of these indices, the CCPR and IEL count show a response which occurs early in the GvHR, and which parallels the proliferative response in the host spleen. These findings confirm and extend an earlier report of an enhanced CCPR in the GvHR in F_1 mice (MacDonald & Ferguson 1977) and support the hypothesis that the CCPR and IEL count are reliable and sensitive parameters of the local CMI occurring in the intestinal mucosa of animals with GvHR. In future studies in this thesis, these indices will be used to identify the presence of mucosal CMI in the jejunum of mice with intestinal immune responses.

The observations that the IEL count and mucosal mast cell infiltration were increased during the GvHR are similar to those made in irradiated F_1 mice with GvHR (Guy-Grand et al 1978). Although the mast cell response was delayed in comparison to the IEL count, the results support the hypothesis that mucosal mast cells are a thymus-dependent population. The present study does not however resolve the controversy as to whether these are descendents of mucosal T cells themselves (Guy-Grand et al 1978).



Age (days)	8	9	11	12	13	15	17	20	27	28	35	70
n GvHR	3	3	3	4	2	3	6	9	4	6	4	5
n Controls	3	3	3	4	4	3	6	7	8	7	4	5

Fig. 6.1. Progress of the GvHR in neonatal (CBA x BALB/c)F_1 mice. Spleen Indices in mice with GvHR and growth rates of GvHR and control mice. Mice were injected with 2.5×10^7 CBA spleen cells or RPMI 1640 at 5-7 days of age. Bars represent means $\pm$ 1 s.d. for each group. Lower panel gives numbers of mice/group and age at time of sacrifice.

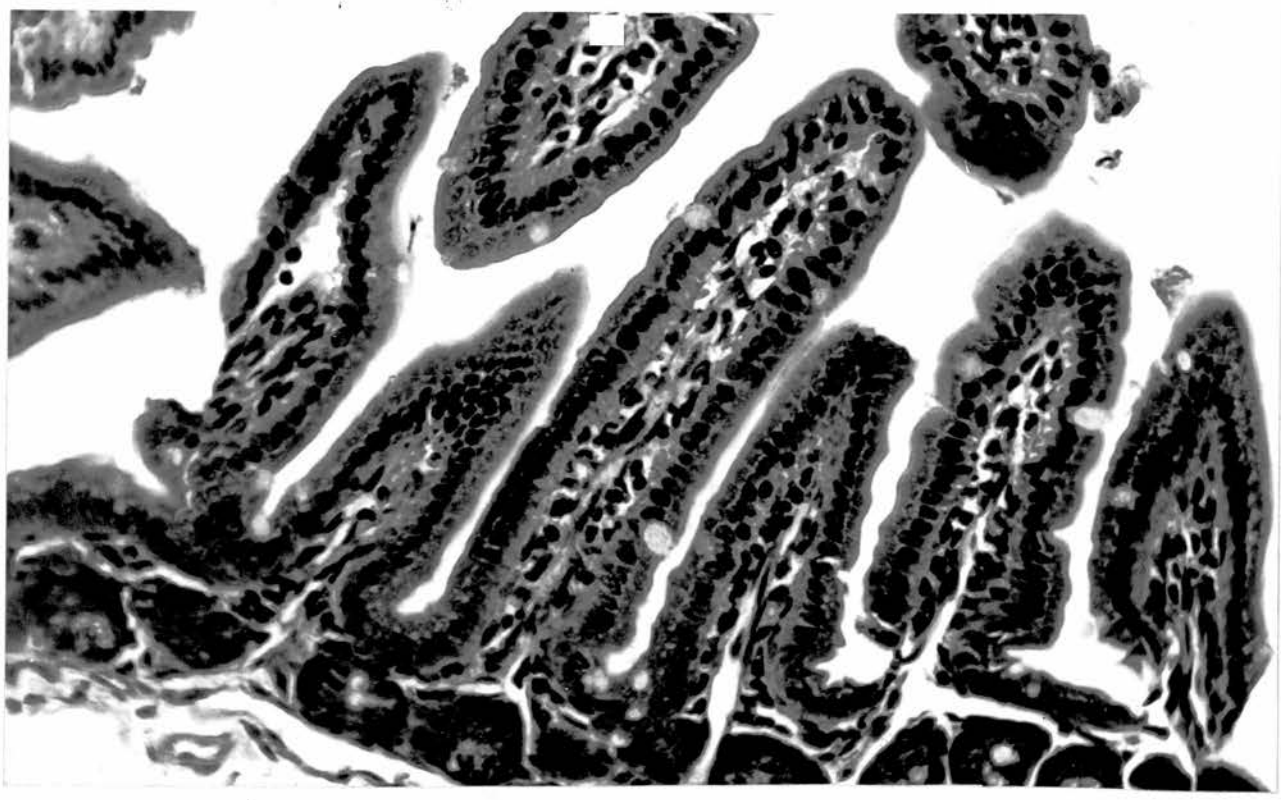


Fig. 6.2. Jejunal morphology in normal (CBA x BALB/c)F_1 mouse aged 17 days. Crypts are short and intraepithelial lymphocytes rarely seen (H & E x 320).

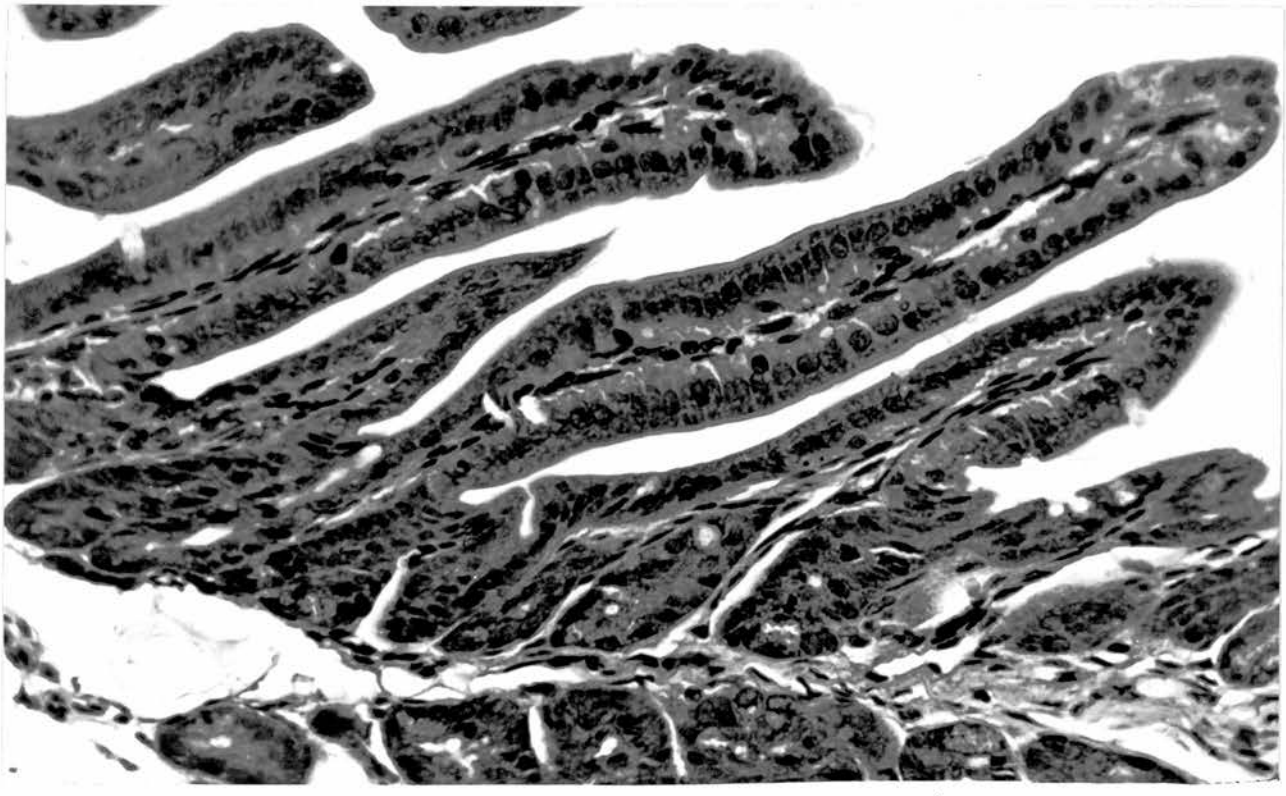


Fig. 6.3. Jejunal morphology in 17 day old (CBA x BALB/c)F_1 mouse 11 days after induction of GvHR. Crypts are longer than in age-matched control (Fig. 6.2) and IEL are more numerous (H & E x 320).

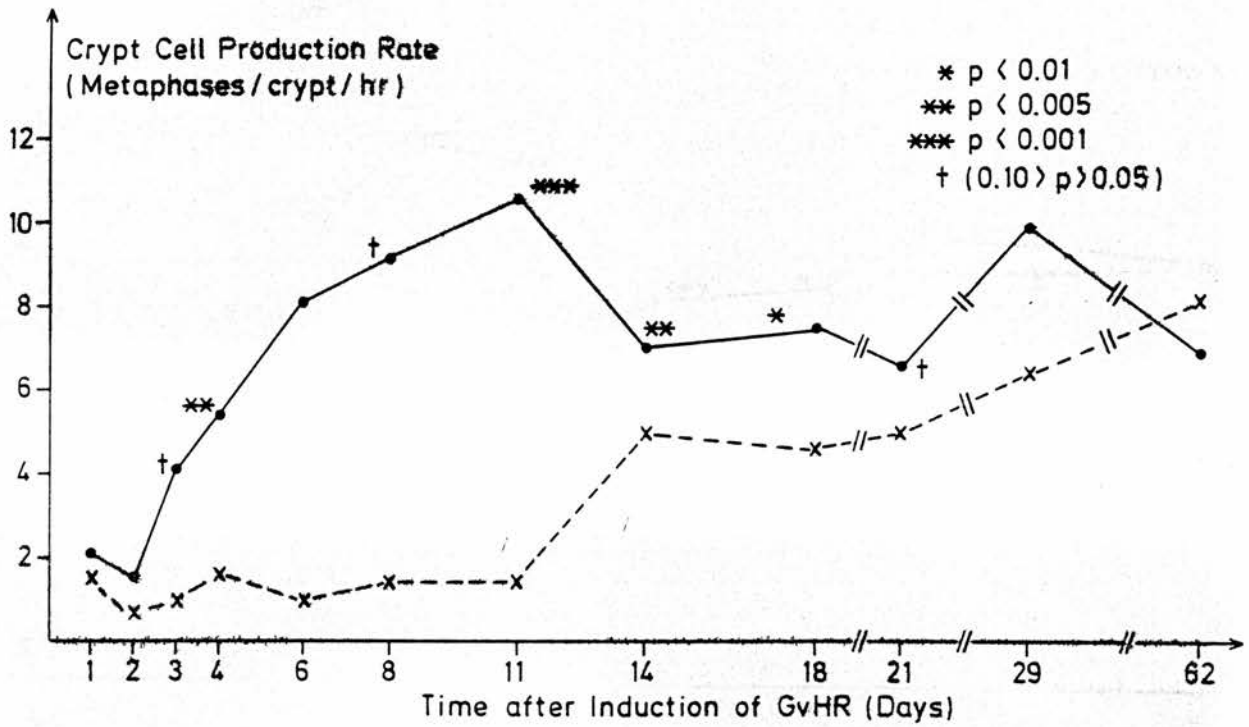


Fig. 6.4. Crypt cell production rate in neonatal (CBA x BALB/c) F_1 mice with GvHR and in littermate controls at intervals after transfer of parental cells.

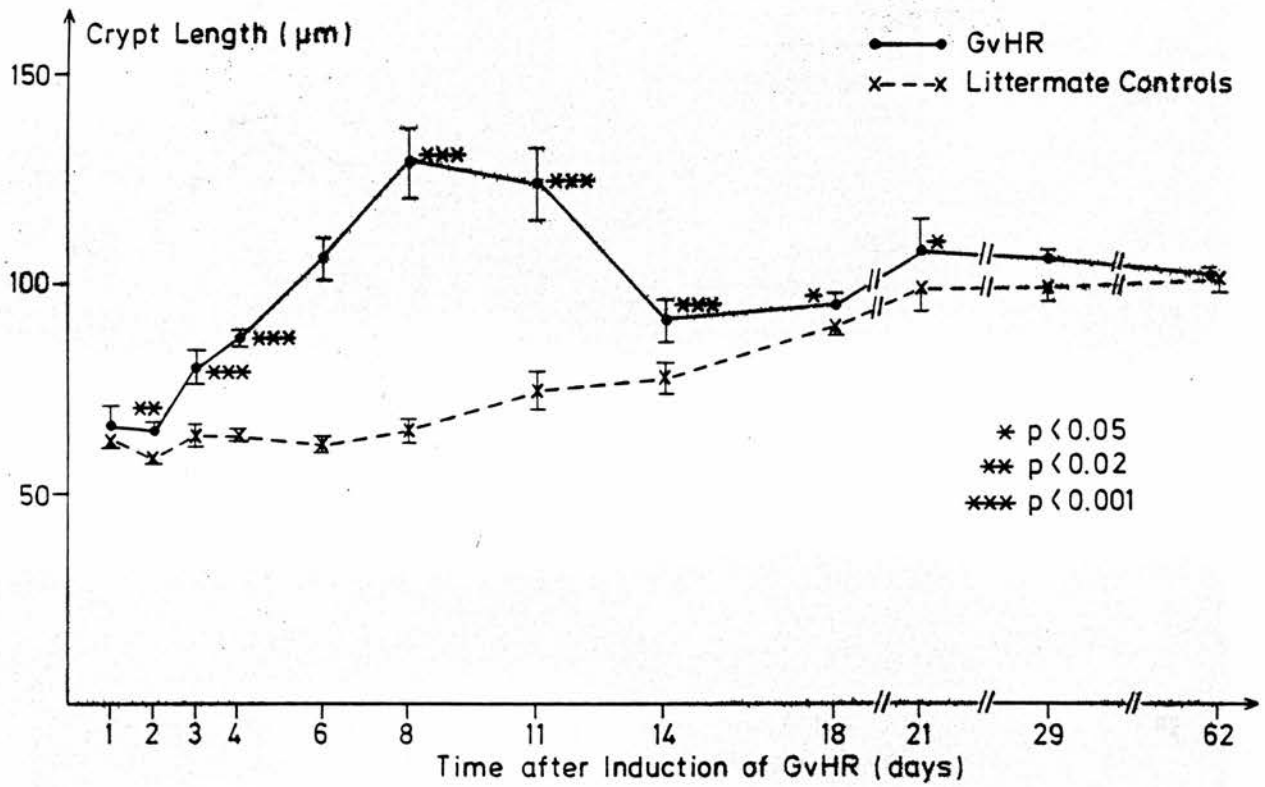


Fig. 6.5. Crypt lengths in neonatal (CBA x BALB/c)F_1 mice with GvHR and in littermate controls. Bars represent mean ± 1 s.d. for each group of mice.

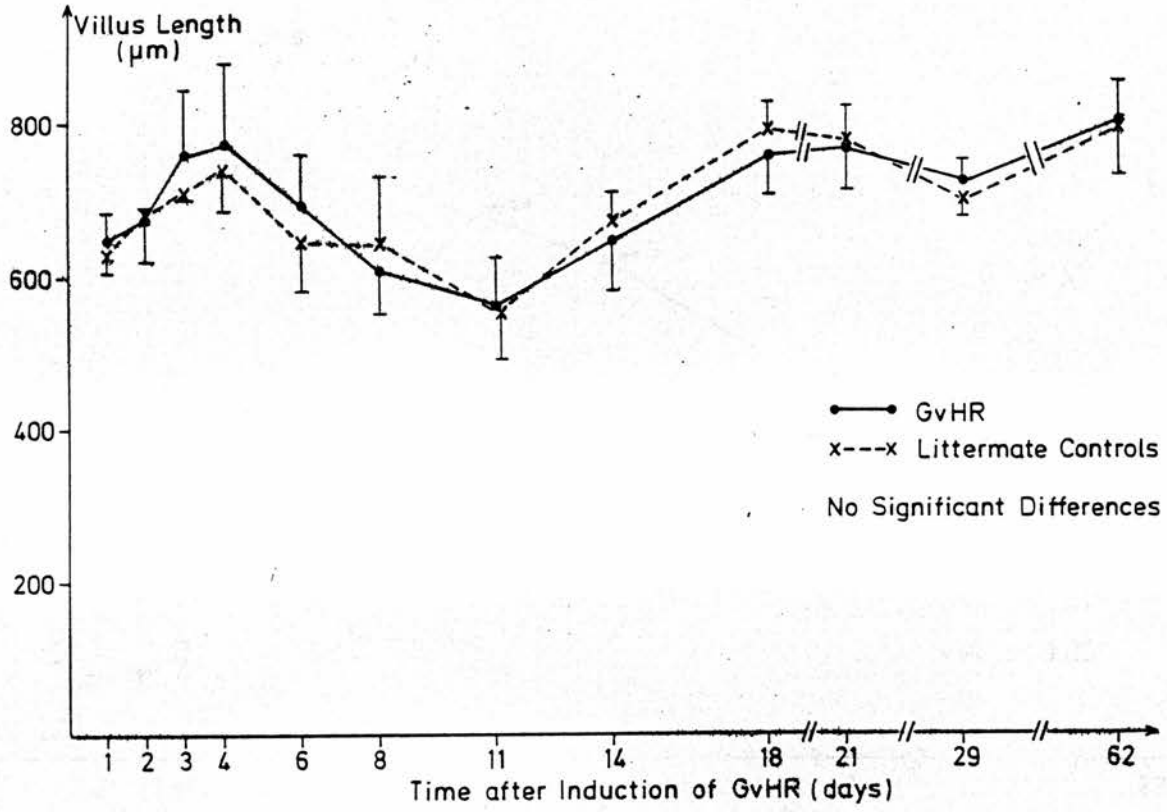


Fig. 6.6. Villus lengths during the GvHR in neonatal (CBA x BALB/c)F_1 mice and in littermate controls. Bars represent mean $\pm$ 1 s.d. for each group.

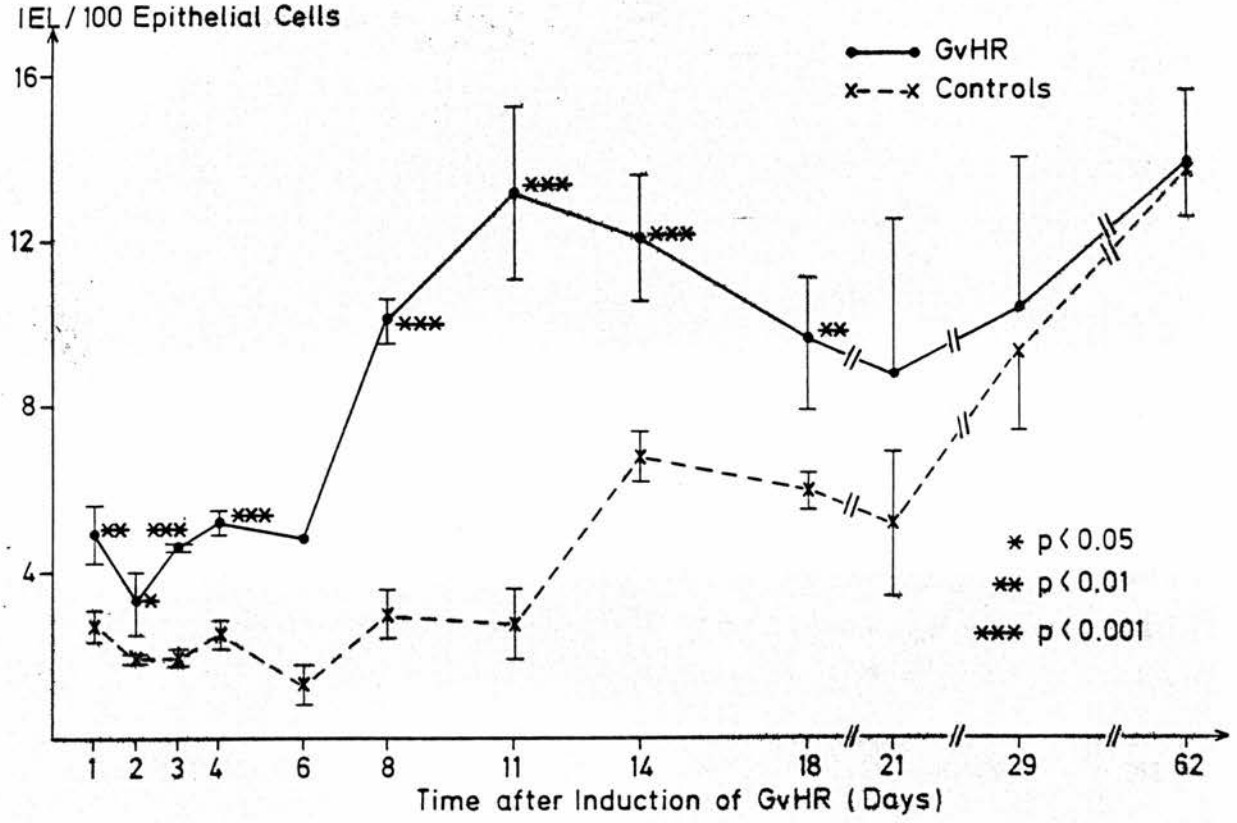


Fig. 6.7. Effect of GvHR on intraepithelial lymphocyte counts in neonatal (CBA x BALB/c)F_1 mice and in littermate controls. Means $\pm$ 1 s.d. for each group.

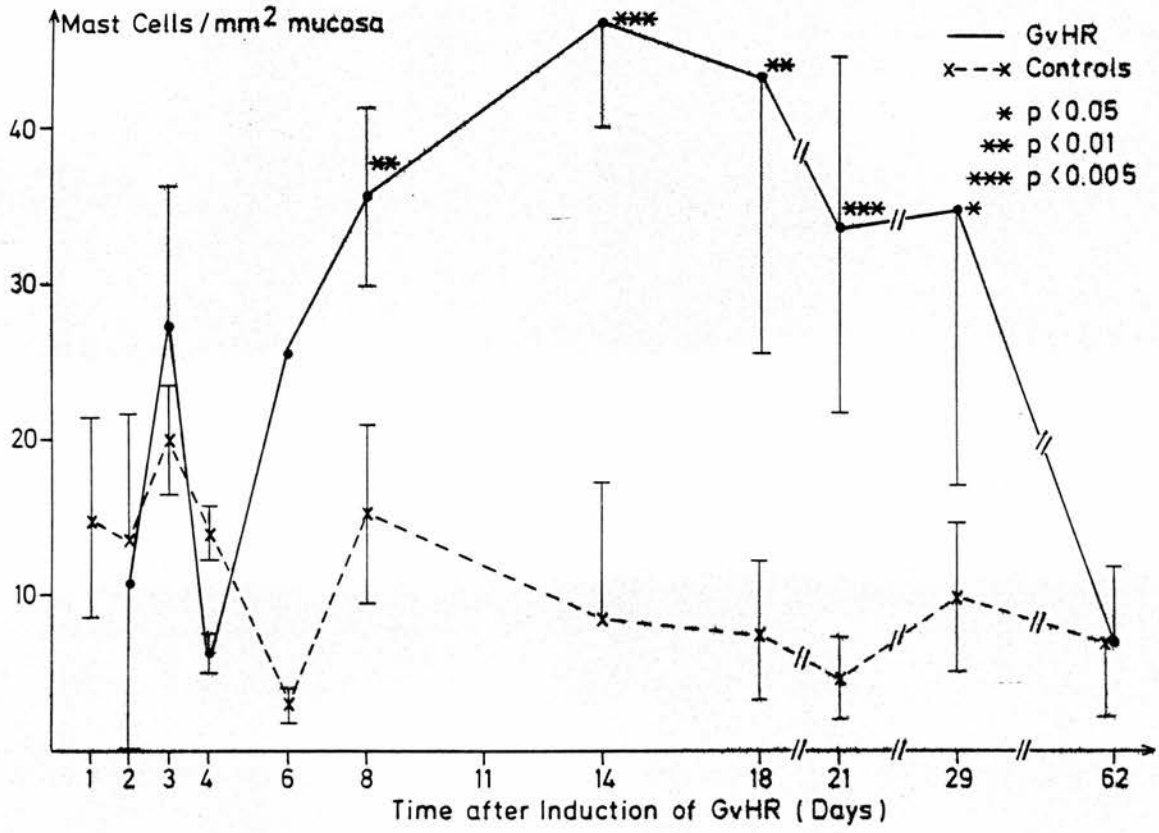


Fig. 6.8. Effect of GvHR on mucosal mast cell counts in neonatal (CBA x BALB/c)F_1 mice and in littermate controls. Means $\pm$ 1 s.d. for each group.

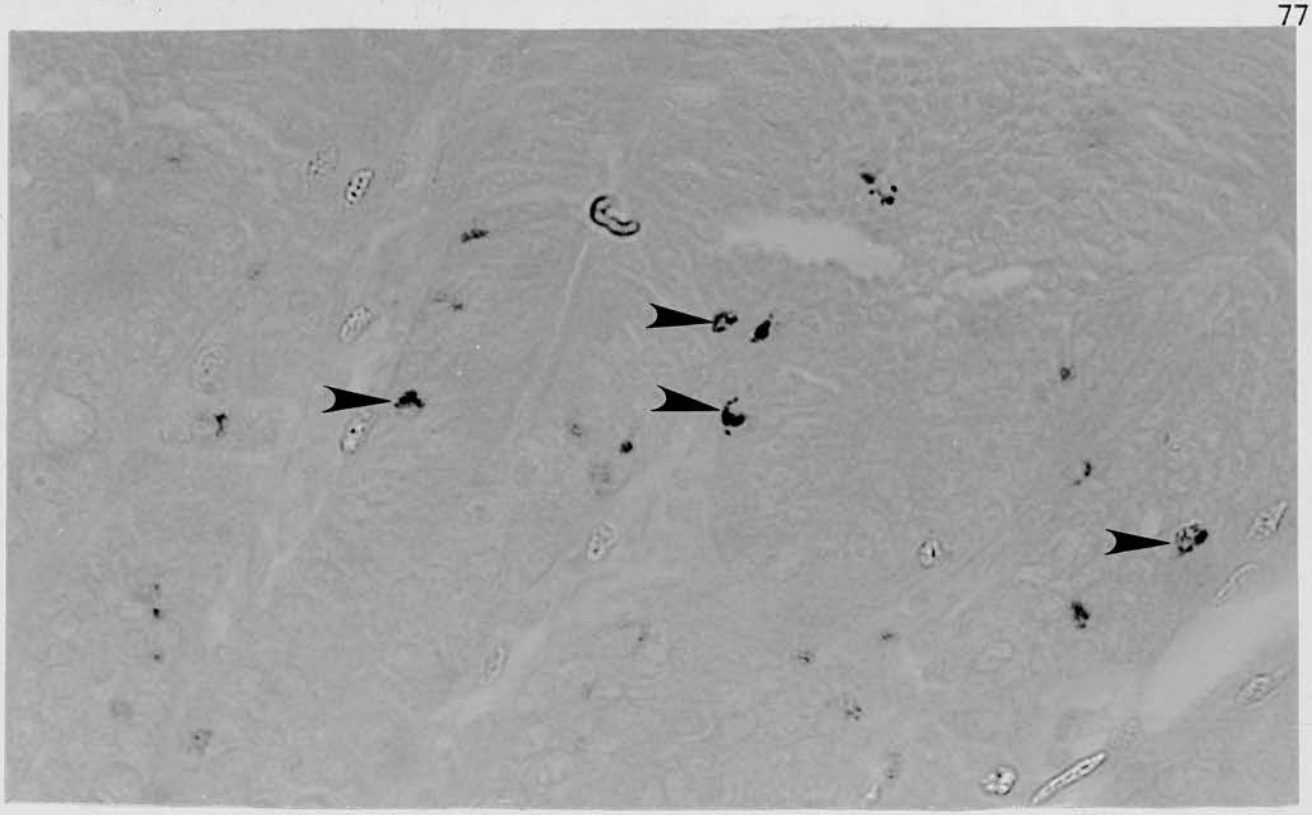


Fig. 6.9. Mucosal mast cells in 27 day old (CBA x BALB/c)F_1 mouse, 18 days after induction of the GvHR (arrows). MMC are found predominantly in the lamina propria round crypts. Similar fields in control mice had 1 or fewer MMC. (Astra Blue/Safranin x 500.)

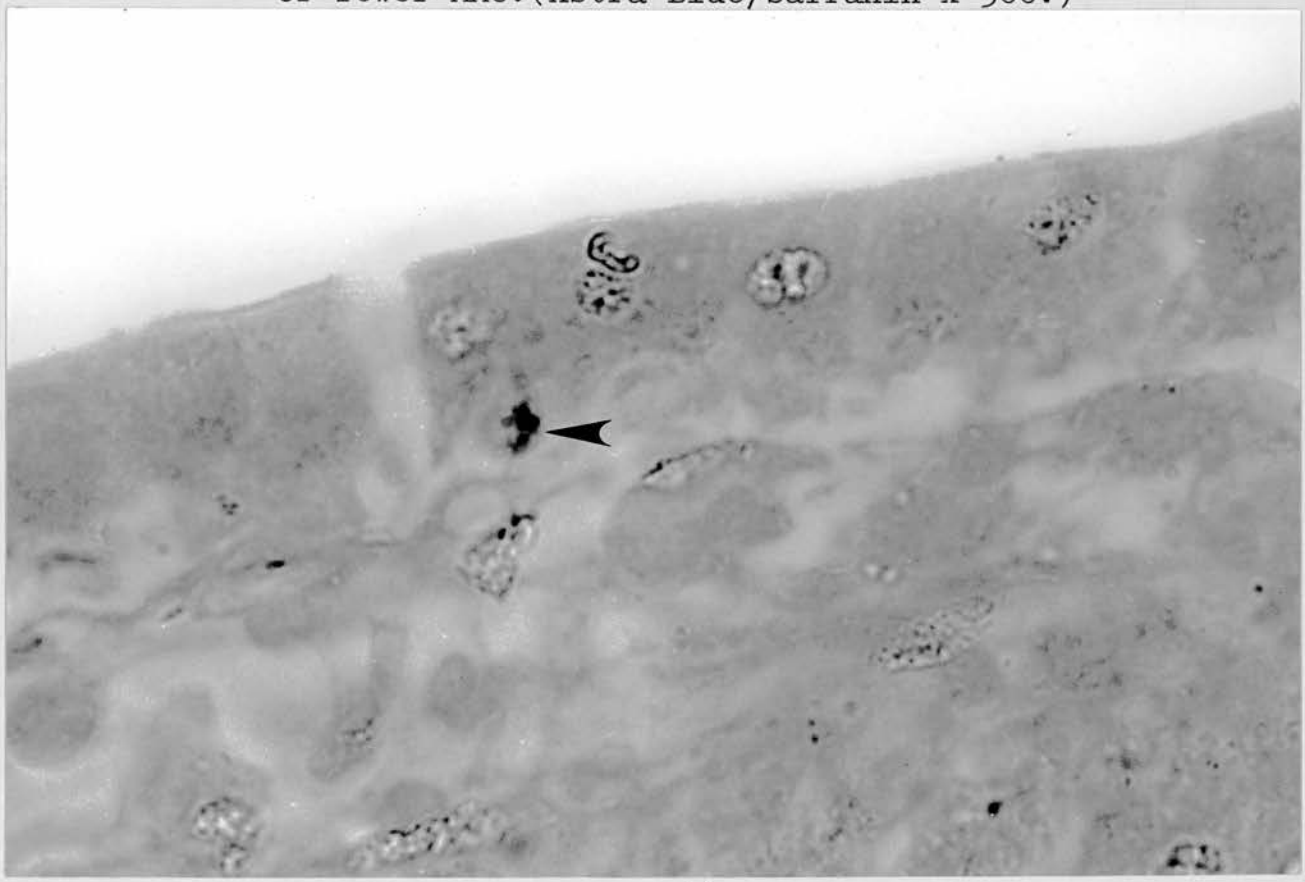


Fig. 6.10. Intraepithelial cell staining positively for mast cell granules in villus of (CBA x BALB/c) mouse shown above (arrow). Cells of this kind were not seen in control mice and granules were smaller than in lamina propria MMC. (Astra Blue/Safranin x 1250.)

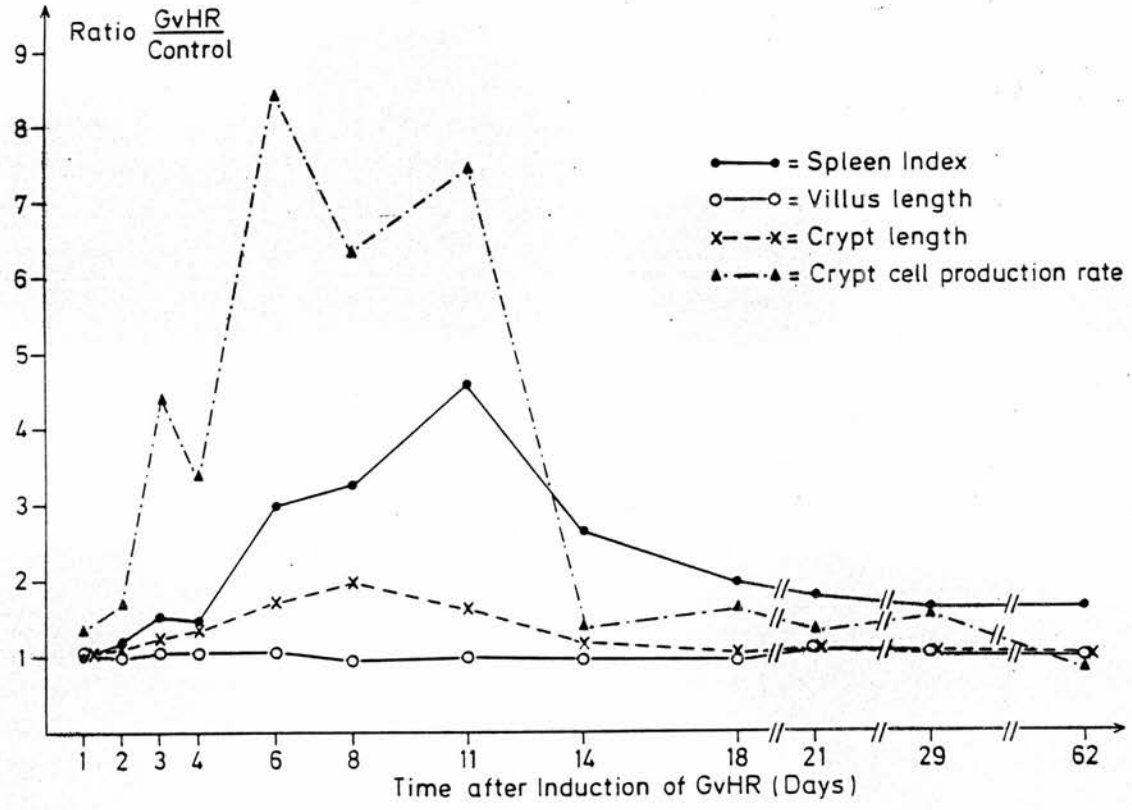


Fig. 6.11. Correlation of induces of mucosal architecture with the Spleen Index throughout the course of the GvHR in neonatal (CBA x BALB/c)F_1 mice. At each time, values for villus length, crypt length and CCPR in mice with GvHR were expressed as a ratio to the value in littermate controls. Crypt length and CCPR closely follow the Spleen Index.

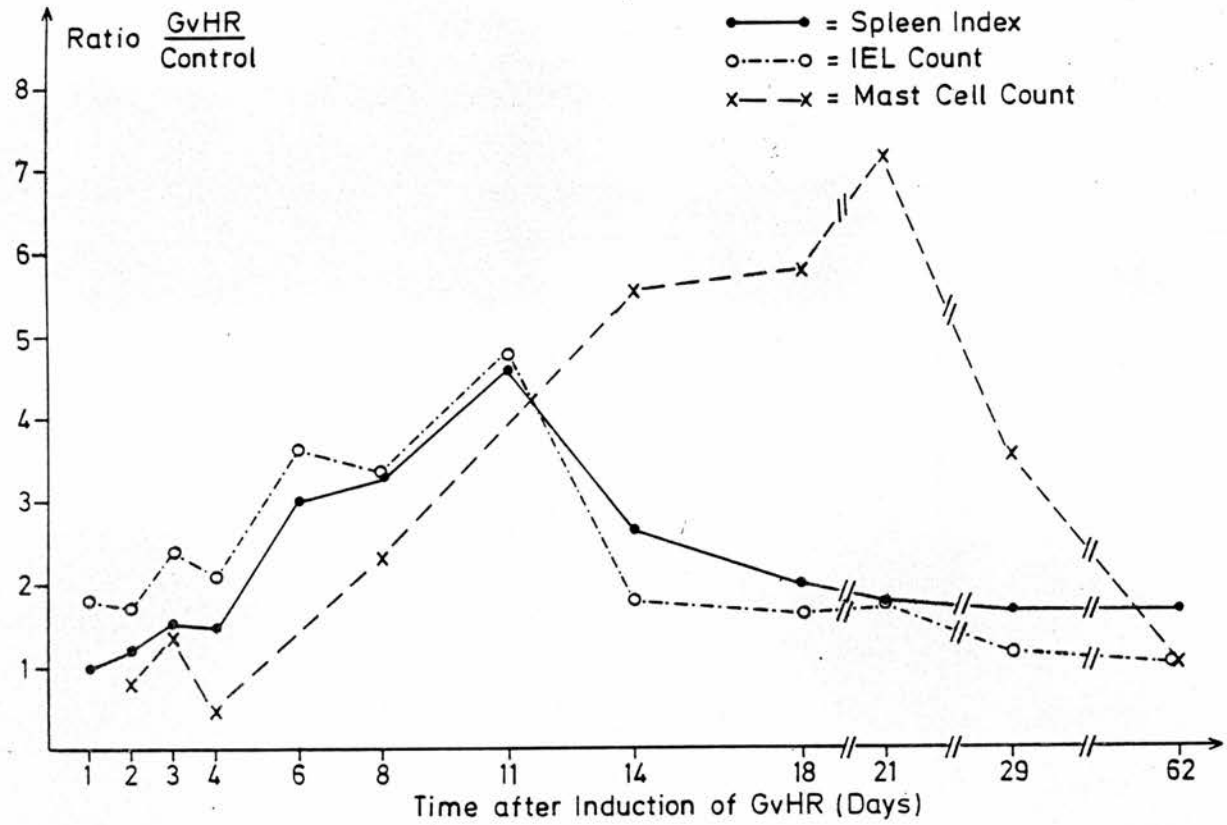


Fig. 6.12. Correlation of cellular infiltrates with the Spleen Index throughout the course of the GvHR. Values for IEL count and MMC count in mice with GvHR are expressed as ratio to control values. Note that the IEL count follows the Spleen Index exactly while the rise in MMC count is delayed and prolonged.

CHAPTER 7

PATHOGENESIS OF THE SMALL INTESTINAL INJURY DURING THE GRAFT-VERSUS-HOST REACTION

Introduction

The results of the previous chapter confirm that the GvHR has significant effects on the small intestinal mucosa of mice and showed that the CCPR and IEL count were closely related to the proliferative response in the GvHR. During the GvHR, there is a large expansion of cytotoxic cells (Cerottini et al 1971; Singh et al 1972; Fung & Sabbadini 1976) and cytotoxic damage to the intestinal epithelium could be responsible for the mucosal features noted earlier. However, villous shortening was not observed in these experiments and in an earlier study, occurred after the stimulation of CCPR was fully developed (MacDonald & Ferguson 1977). These facts strongly indicate that cytotoxicity does not account for the mucosal alterations due to the GvHR, and are consistent with the hypothesis that "enteropathic lymphokines" released during mucosal CMI reactions cause these changes (Ferguson & MacDonald 1977).

The further definition of the mechanisms of intestinal injury in the GvHR was important for my work and the experiments described in this section had two principal objectives; firstly to examine the possibility that soluble factors, released by activated lymphocytes, were responsible for the mucosal injury of the GvHR and secondly to eliminate intestinal bacteria and other local factors as causes of the mucosal changes observed.

Damage to skin (Billingham & Streilen 1968) and kidney (Elkins & Guttman 1968) has been described in the GvHR, even if these tissues are syngeneic to the alloreactive donor

lymphocytes. More recently, this "innocent bystander" phenomenon has been described in grafts of foetal small intestine using measurements of mucosal morphology on conventional histological sections (Elson et al 1977). I have therefore re-examined the "bystander" phenomenon in intestinal mucosa using the CCPR and IEL count as parameters of mucosal CMI, measured in antigen-free heterotopic grafts of parental small intestine implanted in F_1 mice with GvHR. Additional evidence to support a role for soluble mediators was sought by performing these experiments in both an H-2 incompatible and in an H-2 compatible, Mls incompatible strain combination, since cytotoxic cells are not generated by Mls differences (Wolters, Brons, van der Kwast & Benner 1980).

In addition, by using antigen-free grafts of intestine, it is possible to exclude an effect of local intestinal factors on the CCPR and IEL count and this approach should confirm these as sensitive parameters of mucosal CMI itself.

Experimental details

Adult F_1 mice were given bilateral grafts of either F_1 (Groups 1 and 2) or parental-type (Groups 3 and 4) foetal small intestine, implanted under the kidney capsule. These were allowed to grow for 4 weeks and a GvHR was then induced in the host animals of groups 2 and 4 by i.p. injection of 6×10^7 parental spleen cells. Two weeks later, the foetal grafts and pieces of host jejunum were removed for examination. In most cases, the results of mice in control groups 1 and 3 were combined, and results for the host jejunum in groups 2

and 4 were also combined. (Table 7.1).

The numbers of specimens suitable for examination in each group are shown in Table 7.2.

Spleen Indices

The group of (CBA x BALB/c)F_1 animals injected with CBA spleen cells had significantly greater relative spleen weights than controls (58.7 $\pm$ 10.6 mg/10 g body weight vs 40.1 $\pm$ 7.2 mg/10 g weight) giving a Spleen Index of 1.47 ($p < 0.001$) and confirming the presence of the GvHR (Table 7.3). In the H-2 compatible, M1s incompatible (CBA x C3H)F_1 combination, GvHR mice have no increase in relative spleen weight. This concurs with previous findings in GvHR using H-2 compatible combinations (Eichwald, Hart & Eichwald 1969; Cantrell & Hildemann 1972).

It should be noted that none of the animals with GvHR showed any loss of weight.

Mucosal changes during the GvHR

1. Host jejunum

Histology: Conventional H & E sections revealed no differences between the groups and no obvious damage to the mucosa of animals with GvHR.

Microdissection: Significant alterations in mucosal architecture in both (CBA x BALB/c)F_1 and (CBA x C3H)F_1 mice with GvHR were observed by this technique (Fig. 7.1). In (CBA x BALB/c)F_1 mice with GvHR there were significant increases in CCPR (10.6 vs 6.1 $p < 0.02$) and crypt length (124.2 $\pm$ 5.6 mμ vs 109.9 $\pm$ 5.3 mμ $p < 0.005$) compared to controls. In (CBA x C3H)F_1

mice, the GvHR has induced similar increases in both CCPR (7.8 vs 4.9 $p < 0.05$) and crypt length (127.7 $\pm$ 9.2 mμ vs 109.7 $\pm$ 6.9 mμ $p < 0.001$). In neither experiment were alterations in villous length observed.

Intraepithelial lymphocyte counts: Fig. 7.2 shows that in (CBA x BALB/c)F_1 mice with GvHR, the IEL count was increased to 16.0 $\pm$ 1.2 IEL/100 Epithelial cells compared to controls 9.9 $\pm$ 2.1 IEL/100 Epithelial cells ($p < 0.001$). Similarly, (CBA x C3H)F_1 mice with GvHR had 20.5 $\pm$ 2.5 IEL/100 Epithelial cells compared to 14.2 $\pm$ 1.6 IEL/100 Epithelial cells in controls ($p < 0.001$).

Disaccharidase levels: In (CBA x C3H)F_1 mice with GvHR, there was a mild, but insignificant fall in jejunal lactase activity compared to controls (2.0 $\pm$ 1.0 vs 2.9 $\pm$ 1.9 mmol lactose/min/g). Sucrase levels were identical in both groups (12.3 $\pm$ 2.5 vs 11.8 $\pm$ 4.6 mmol sucrose/min/g).

2. Allografts and isografts of foetal gut implanted in control animals

The growth and development of heterotopic grafts of foetal gut has been described elsewhere (Ferguson & Parrott 1972b; Ferguson 1973), where it has been shown that these grafts develop mucosal structures of normal appearance.

Histology: When removed 6 weeks after implantation, grafts were distended by collections of extruded matter, but histology confirmed that the majority of grafts were morphologically intact with growing mucosa at the time of sacrifice. (Fig. 7.5).

Microdissection: As described previously, these antigen-free grafts have lower values than adult jejunum for all indices (Fig. 7.3). There were however, no differences between F_1 type grafts (Group 1) and parental-type grafts (Group 3) implanted in control animals in either experiment, and these results were therefore combined for comparison with results from GvHR animals.

Intraepithelial lymphocyte counts: Once again results from groups 1 and 3 are combined since there were no differences between parental and F_1 grafts (Fig. 7.4). In addition, the values were low compared to the host jejunum with means of 3.7 ± 0.8 IEL/100 Epithelial cells in grafted (CBA x BALB/c)F_1 mice and 2.4 ± 0.6 IEL/100 Epithelial cells in grafted (CBA x C3H) F_1 animals. This again confirms earlier findings (Ferguson and Parrott 1972a).

3. Foetal gut grafts in animals with GvHR

Histology: Once again, the majority of grafts had viable mucosa and neither graft type showed microscopic evidence of mucosal damage. However, when serial sections through the entire length of each graft type from (CBA x BALB/c)F_1 mice with GvHR were examined, there appeared to be an increased frequency of Lymphoid follicles in both F_1 and parental grafts compared to grafts in control animals (Table 7.4 Fig. 7.6) Thus, four lymphoid follicles were seen in 10 grafts from control mice while five follicles were seen in six F_1 grafts and five in 10 parental grafts in GvHR mice. The differences between control mice and mice with GvHR were not however statistically significant (X^2 test).

Microdissection: The values for the different parameters of mucosal architecture in grafts in groups 1 and 3 (Controls), group 2 (F_1 grafts + GvHR) and group 4 (Parental grafts + GvHR) are shown in Fig. 7.3. In the (CBA x BALB/c)F_1 combination, there was significant lengthening of the crypts in both F_1 type (Group 2 118.2 $\pm$ 15.6 mμ $p < 0.001$) and parental type grafts (Group 4 109.6 $\pm$ 10.6 mμ $p < 0.001$) compared to controls (93.7 $\pm$ 3.9 mμ), while the CCPR was also raised in F_1 grafts (Group 2 8.6 $p < 0.01$) and in parental grafts (6.2 $p < 0.05$), compared to the value of 3.1 for controls. Villus lengths remained normal.

In (CBA x C3H)F_1 mice, the results were similar. Crypt lengths increased during the GvHR in both F_1 type (Group 2 111.9 $\pm$ 17.1 mμ $p < 0.02$) and parental-type grafts (Group 4 120.5 $\pm$ 22.9 mμ $p < 0.005$) compared to controls (98.0 $\pm$ 7.2 mμ). There was a concomitant increase in CCPR in parental type grafts (6.5 $p < 0.10$) compared to 4.0 for controls. On this occasion, F_1 type grafts did not have an increased CCPR (3.3). Again no villous abnormalities were observed.

Intraepithelial lymphocyte counts: Once again, the GvHR in the host animals had similar effects on both graft types with respect to the IEL count (Fig. 7.4). In (CBA x BALB/c)F_1 animals, F_1 grafts (Group 2) showed an increase in IEL count to 6.9 $\pm$ 1.0 IEL/100 Epithelial cells compared to 3.7 $\pm$ 0.8 IEL/100 Epithelial cells in controls ($p < 0.001$). Parental-type grafts (Group 4) had a similar, highly significant rise in IEL count (7.2 $\pm$ 1.1 IEL/100 Epithelial cells $p < 0.001$).

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Fig 7.4: In both experiments, the results for Group 2 have been plotted above Group 4, and vice versa.

An identical pattern was observed in (CBA x C3H)F_1 mice during the GvHR. Here, F_1 grafts (Group 2) had an increased IEL count of 5.3 $\pm$ 0.5 IEL/100 Epithelial cells ($p < 0.001$). In this case, controls had 2.4 $\pm$ 0.6 IEL/100 Epithelial cells.

Conclusions

In summary, these experiments have shown that, as in neonatal F_1 mice injected with parental spleen cells, there are increases in CCPR, crypt length and IEL count in adult F_1 mice with GvHR. Similar changes were also observed in antigen-free grafts of foetal gut of both F_1 and parental-type when the host animal had GvHR.

Thus, these experiments have confirmed the CCPR and IEL count to be reliable and sensitive indices of the mucosal phase of the GvHR. In adult F_1 mice with a moderate form of GvHR and little evidence of intestinal injury as shown by histology or epithelial enzyme levels, these parameters showed consistent and highly significant increases in the jejunum of both (CBA x BALB/c)F_1 and (CBA x C3H)F_1 mice. Furthermore, identical alterations were also found in the mucosa of F_1 foetal gut grafts during the GvHR, despite the fact that these grafts are sterile and antigen-free. Thus, although intestinal bacteria have been implicated in the lesions of the GvHR (Van Bekkum & Knaan 1977), it is apparent that alterations in CCPR and IEL count are not dependent on direct contact with a local bacterial flora. These findings are strong support for the hypothesis that alterations in

CCPR and IEL count are direct consequences of mucosal CMI.

In addition, the results of this chapter are consistent with the idea that soluble factors are responsible for the mucosal changes in CMI reactions (Ferguson & MacDonald 1977). Parental-type grafts of foetal gut were damaged during the GvHR despite being syngeneic to the injected donor cells and identical mucosal changes were found in both host jejunum and foetal gut grafts during the GvHR in the M1s incompatible, (CBA x C3H) strain combination. Direct cytotoxic action by donor cells is therefore unlikely to be responsible for the mucosal lesions observed.

EXPERIMENTAL GROUPS						HOST EFFECT
1	Adult F_1 mice with bilateral grafts of F_1 foetal small intestine	4 weeks	6×10^7 F_1 spleen cells I.P.	2 weeks	All mice given 7.5 mg/kg colchicine I.P. 1/2 - 2 hr before sacrifice. Body, spleen weights measured.	Normal
2			6×10^7 parental spleen cells I.P.			GvHR
3	Adult F_1 mice with bilateral grafts of parental strain foetal small intestine	4 weeks	6×10^7 F_1 spleen cells I.P.	2 weeks	Host jejunum and one graft fixed in formalin and in ethanol/ acetic acid	Normal
4			6×10^7 parental spleen cells I.P.			GvHR

Table 7.1. General protocol of experiments on effects of Graft-versus-Host Reaction on the intestinal mucosa of adult F_1 mice.

	EXPERIMENTAL GROUPS	TOTAL NO. OF MICE	HOST JEJUNUM MICRODISSECTION	HOST JEJUNUM IEL	FOETAL GRAFTS MICRODISSECTION	FOETAL GRAFTS IEL
CBA ⟶ (CBA x BALB/c)F_1 Experiments	1	10	10	5	9	5
	2	10	10	5	10	6
	3	9	9	5	9	5
	4	10	10	5	8	10
CBA ⟶ (CBA x C3H)F_1 Experiments	1	10	10	5	7	5
	2	10	10	5	8	10
	3	10	10	5	7	6
	4	10	10	5	7	7

N.B. For some analyses groups 1 & 3 and 2 & 4 were combined.

Table 7.2. Number of specimens of host small intestine and foetal grafts used for mucosal measurements and intraepithelial lymphocyte counts.

EXPERIMENTAL GROUPS	BODY WEIGHTS	SPLEEN WEIGHTS	RELATIVE SPLEEN WEIGHTS (mg/10g body wt.)	SPLEEN INDEX
CBA ⟶ (CBA x BALB/c)F_1				
Controls (Gps 1 & 3)	25.2 ± 2.60 g	101.2 ± 18.1 mg	40.10 ± 7.17	1.47
GvHR (Gps 2 & 4)	25.2 ± 2.06 g	147.8 ± 26.7 mg*	58.73 ± 10.62*	
		* $p < 0.001$		
CBA ⟶ (CBA x C3H)F_1				
Controls	23.8 ± 2.42 g	83.2 ± 16.2 mg	34.94 ± 6.8	0.92
GvHR	27.1 ± 2.67 g	86.9 ± 13.0 mg	32.09 ± 4.8	

Table 7.3. Measurements of body and spleen weights (means ± 1 S.D.) in F_1 mice receiving F_1 or parental spleen cells.

EXPERIMENTAL GROUPS	NO. OF LYMPHOID FOLLICLES/GROUP
F_1 and Parental Grafts in Control Mice (1+3)	4/10
F_1 Grafts and GvHR Mice (2)	5/6
Parental Grafts in GvHR Mice (4)	5/10

Table 7.4. Number of lymphoid follicles seen in serial sections of foetal grafts implanted in (CBA x BALB/c) F_1 mice injected with 6×10^7 F_1 or CBA spleen cells.

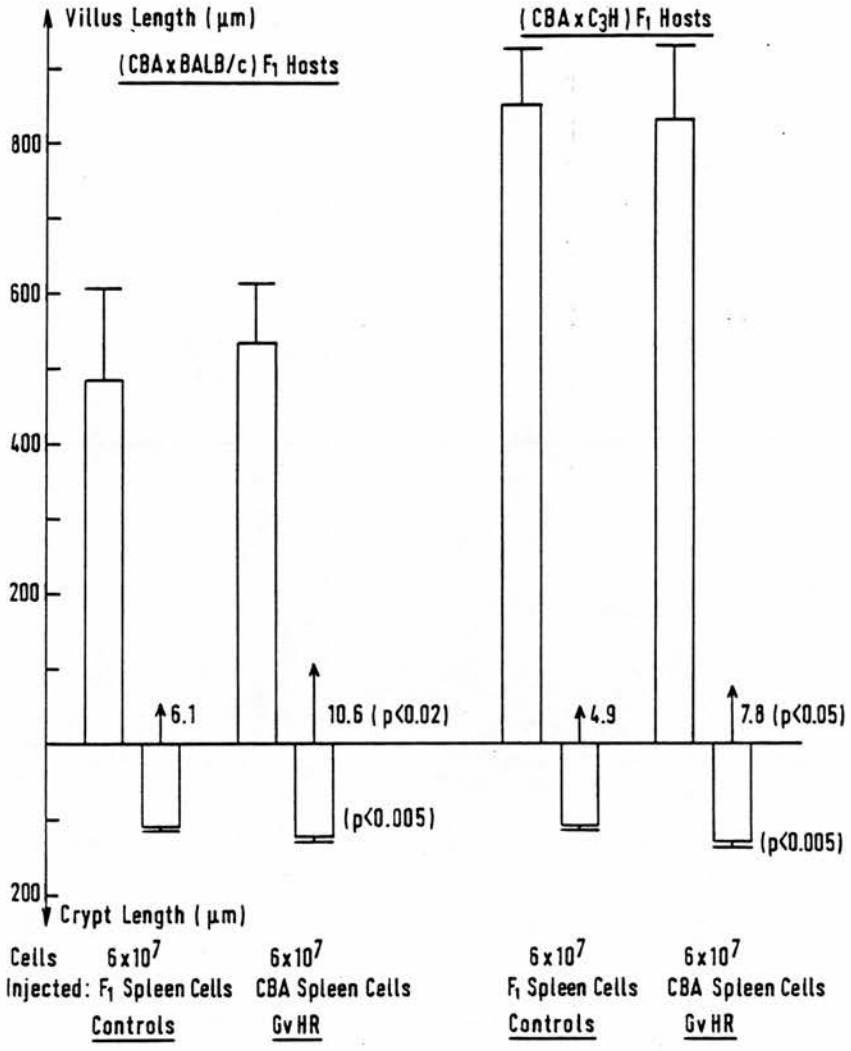

Fig. 7.1. Effect of the GvHR on mucosal architecture in the jejunum of adult (CBA x BALB/c)F_1 and (CBA x C3H)F_1 mice. Villus length, crypt length and CCPR 14 days after injection of 6 x 10^7 parental (GvHR) or F_1 spleen cells (controls). Bars represent means $\pm$ 1 s.d. for villus/crypt length and arrows show CCPR (19-20 mice/group).

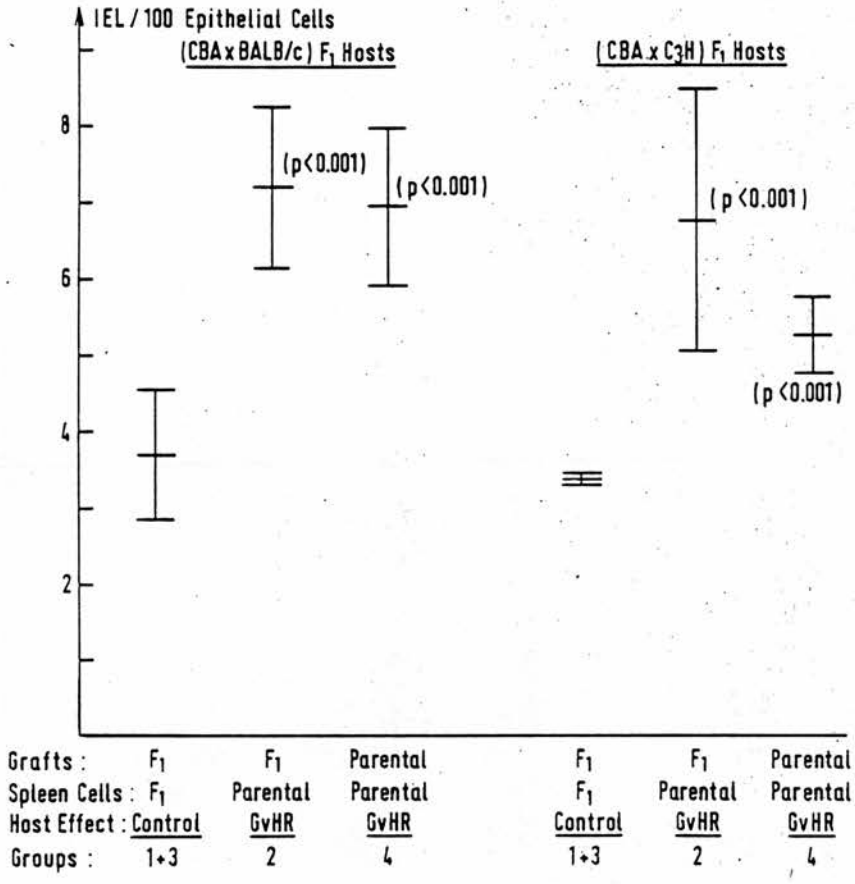

Fig. 7.2. Effect of the GvHR on intraepithelial lymphocyte counts in the jejunum of (CBA x BALB/c)F_1 and (CBA x C3H)F_1 mice. Results are mean IEL count $\pm$ 1 s.d. 14 days after injection of 6 x 10^7 parental (GvHR) or F_1 spleen cells (controls). (20 mice/group).

ERRATUM pp 94 and 96
The graphs for Figs 7.2 and 7.4 have been transposed.
These should be on pages 94 and 96 respectively.

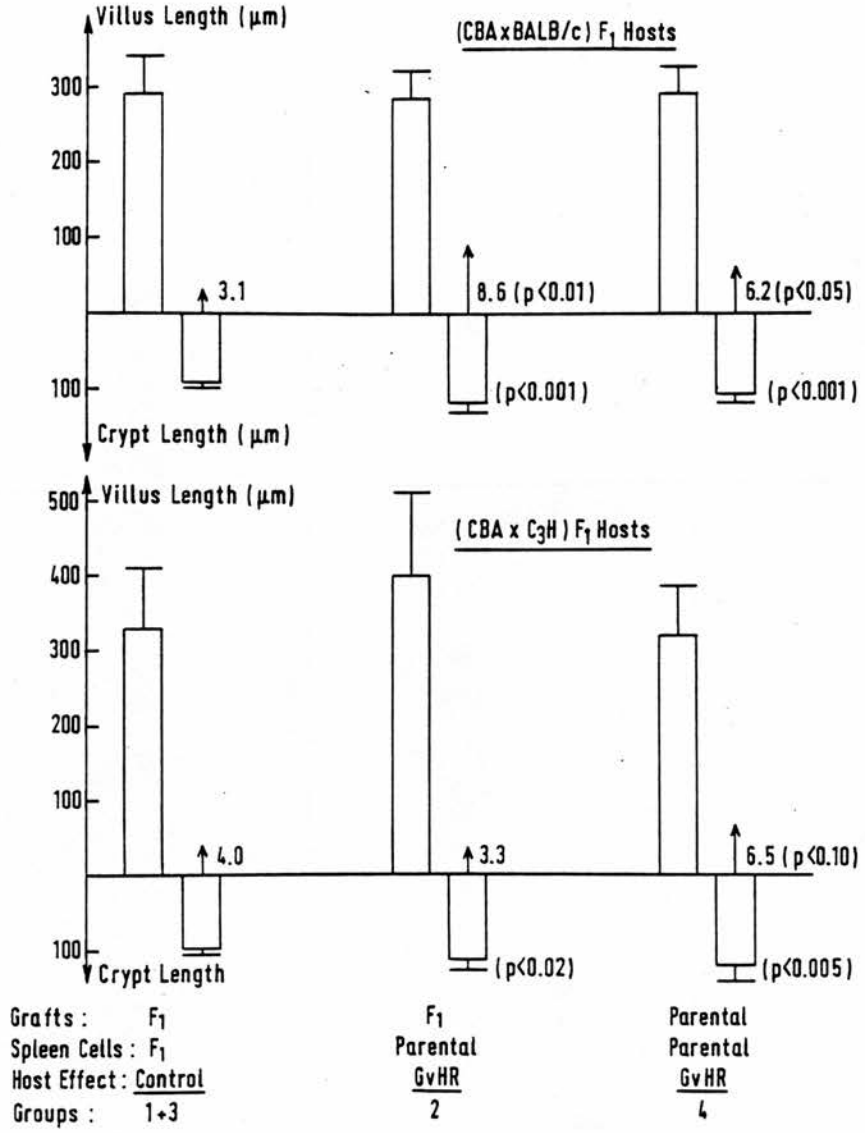

Fig. 7.3. Effect of the GvHR occurring in host animals on the mucosal architecture of F_1 and parental-type grafts of foetal gut. Villus length, crypt length and CCPR in grafts of foetal gut implanted in GvHR mice and implanted in control mice. Bars represent means + 1 s.d for villus/crypt length and arrows show CCPR 14 days after induction of the GvHR. 7-10 grafts were examined in GvHR mice and at least 14 grafts in controls.

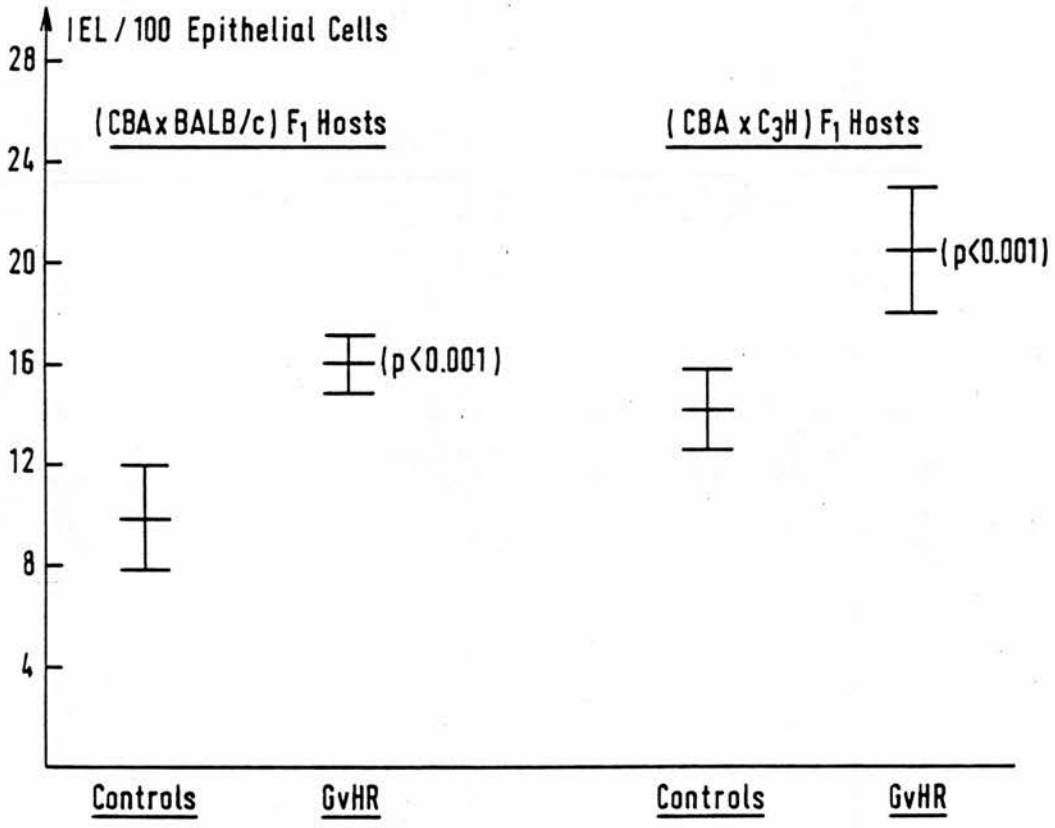

Fig. 7.4. Effect of the GvHR occurring in host animals on the IEL count in F_1 and parental-type grafts of foetal gut. Results are mean IEL count $\pm$ 1 s.d. in grafts implanted in GvHR mice and implanted in control mice; 14 days after induction of the GvHR. 6-10 grafts were examined in GvHR mice and 10 grafts in controls.

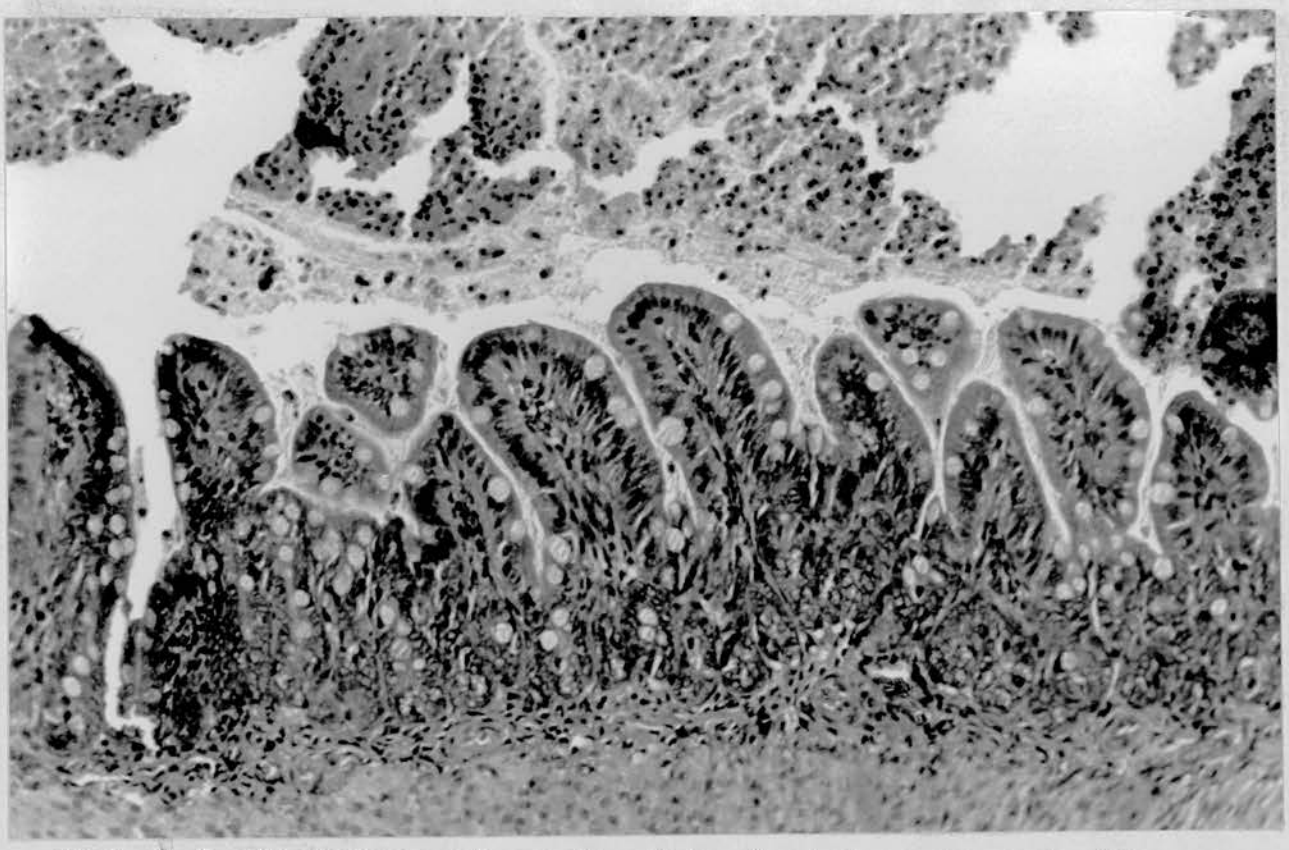

Fig. 7.5. Histology of graft of F_1 foetal small intestine 42 days after implantation under the kidney capsule of a control (CBA x C3H)F_1 mouse. The mucosal appearances were normal in these grafts, although the values for all indices were lower than in normally sited intestine (H & E x 160).

Fig. 7.6. Lymphoid follicle in graft of F_1 gut implanted in (CBA x BALB/c)F_1 mouse with GvHR (H & E x 50).

CHAPTER 8

MIGRATION INHIBITION OF LYMPH NODE LYMPHOCYTES AS AN ASSAY FOR CM^{I}

The CCPR and IEL count have been shown to be sensitive and reliable indices of a known CMI reaction occurring in the intestinal mucosa. However, these have the disadvantage of being indirect parameters of the immune response and in addition, may be affected by many non-immunological factors. Since my aim was to induce local intestinal cell mediated immunity to a dietary antigen, it was important to be able to monitor local immunity by an in vitro immunological test which would complement mucosal measurements in vivo.

In vitro assays of CMI are of two main types: those involving antigen-induced transformation of sensitised lymphocytes and those which depend on the release of lymphokines from antigen-stimulated lymphocytes (Bloom 1971). For practical reasons of cost and culture facilities, I decided to use a migration inhibition technique. In the mouse, migration inhibition tests have normally been performed with a mixture of the sensitised cells and a migrating indicator cell population. However, a recent report describing a direct migration inhibition test using thymocytes from immunised mice as an in vitro test for DTH (Hughes et al 1980) prompted my attempt to use mouse lymph node cells in a direct inhibition assay.

The object of this group of experiments was therefore to validate migration inhibition of lymph node cells as an in vitro correlate of DTH in vivo by using lymph nodes draining the footpads of mice immunised there with antigen

in adjuvant. Ultimately, my intention was to employ the mesenteric lymph nodes of mice immunised orally with a dietary protein.

Outline of experiments

Lymph node cells were obtained from the popliteal and axillary lymph nodes of BALB/c mice at intervals after intradermal immunisation with OVA in adjuvant into each footpad. In this way, large numbers of sensitised lymphoid cells could be obtained from each mouse. Pooled cells from the lymph nodes of 2-3 mice were used in each test.

Determination of time course and dose dependence of migration inhibition

Initial experiments indicated that lymph node cells would migrate well from capillary tubes and that migration inhibition could be observed after overnight incubation with antigen. I next performed a series of experiments to define optimum culture conditions suitable for routine use.

Fig. 8.1 shows the development of migration inhibition in lymph nodes taken from mice at intervals after immunisation with 100 µg OVA in CFA, assayed with 0.01, 0.1, 1, 10 and 50 mg/ml OVA in the culture medium. Several points emerged from these results. Firstly, 50 mg/ml OVA produced inhibition of migration in all cases and this is likely to be due to a cytotoxic action of this high concentration of OVA. This was not seen at lower concentrations of antigen. No migration inhibition was observed with cells taken 1 week after immunisation, but by 2 weeks there was inhibition in the

wells containing 0.1 mg/ml OVA (M.I. = 0.78), although this was not statistically significant.At 3 weeks, statistically significant inhibition was observed with all test concentrations of OVA. It was found that 0.1 mg/ml OVA gave the best sensitivity and reproducibility (M.I. = 0.49 $\pm$ 0.10) with good results also being obtained with 1 mg/ml OVA (M.I. = 0.66 $\pm$ 0.17). Significant inhibition was found with all concentrations of OVA used at 4 weeks, at a level similar to that observed at 3 weeks. Even by 9 weeks after immunisation, significant migration inhibition was obtained with 0.01 mg/ml OVA (M.I. = 0.53 $p < 0.01$) and 10 mg/ml OVA (M.I. = 0.56 $p < 0.01$

In subsequent experiments, I therefore used 0.1 and/or 1 mg/ml OVA as the concentrations of antigen in inhibition assays.

Specificity of migration inhibition

This was investigated in two ways. Firstly, lymph node cells from OVA immunised mice were migrated in the presence of HSA and secondly, direct inhibitory effects of the antigens were excluded by testing cells from mice immunised with saline in CFA against different concentrations of OVA and HSA. 0.1 mg/ml and 1 mg/ml HSA did not produce significant migration inhibition of cells from OVA immunised mice (Fig. 8.2: M.I. = 0.98 $\pm$ 0.09 and 0.89 $\pm$ 0.01 respectively) and this figure also shows that cells from saline immunised mice were not inhibited by either OVA or HSA at concentrations of 0.1 or 1 mg/ml. In other experiments OVA did not inhibit migration of saline immunised cells at any time after

immunisation except when the highest, toxic concentrations of antigen were used.

Correlation of migration inhibition with systemic immunity

In these experiments, mice were immunised with either 100 µg or 10 µg OVA in CFA or with 100 µg OVA in Incomplete Freund's Adjuvant distributed between three footpads. The animals were tested for haemagglutinating antibodies, systemic DTH and migration inhibition in the presence of 0.1 mg/ml OVA at intervals thereafter. The results are shown in Fig. 8.3.

When mice were immunised with 100 µg OVA in CFA, humoral antibodies were not consistently detected until 2 weeks and a further small increase was seen at 3 weeks. A similar pattern was found with DTH, as measured by the specific increase in footpad thickness 24 hours after an intradermal injection of 100 µg OVA in saline, which as I have found in other experiments, was fully developed by 3 weeks. As shown here and in Fig. 8.1, migration inhibition was also fully developed only by 3 weeks after immunisation, and it is apparent that all parameters were in step in this instance.

A different pattern emerged after immunisation with 10 µg OVA in CFA however. In this case, no animals were found to have antibodies at 1 week, but there was a good antibody response 2 and 3 weeks after immunisation. In contrast excellent systemic DTH could be elicited at 1 week in these animals and it thereafter declined to low levels. Migration inhibition was already significant by 1 week after immunisation with 10 µg OVA (M.I. = 0.72) and continued to develop to a high level thereafter (M.I. = 0.18, 3 weeks).

Thus, immunisation with 10 µg OVA in CFA produced systemic DTH and migration inhibition 1 week afterwards in the absence of antibody formation, although migration inhibition continued to develop while DTH declined in vivo.

Finally, mice were tested for these parameters 3 weeks after immunisation with 100 µg OVA in IFA. These mice have high titres of antibody at this time, but have systemic DTH responses which are substantially less than other groups. No migration inhibition could be elicited in the lymph nodes of these mice, despite the very high Ab levels.

Identification of migrating cells

It is usually assumed that cells migrating from cultures of lymphoid tissues are predominantly macrophages or polymorphs, while lymphocytes are the cells responsible for the production of factors which modulate this activity. The large numbers of lymph node cells which appeared to migrate in the cultures here, led me to believe that the migrating cell was predominantly a lymphocyte. This was confirmed when Giemsa-stained preparations of cells which had migrated from the capillary tubes were examined. It can be seen from Fig. 8.4 that these cells are mostly lymphocytes with only occasional macrophages and other cells to be seen. Furthermore, when migrated cells were allowed to adhere to glass coverslips, Giemsa staining revealed only a small number of glass-adherent cells.

Similar results were obtained when cells were examined by fluorescent labelling for T and B cell markers. Thus, 55%

of cells were positive for Thy 1.2 alone and 37% positive for sIg, with the remaining 8% negative for both markers (Table 8.1). Proportions of these cells in the lymph node cell population before migration were 46% T cells, 36% B cells and 18% other cells. Lymphocytes are therefore the predominant migrating cell type in the migration assay.

Migration characteristics of individual lymphocyte populations

Having observed that lymphocytes were the predominant migrating cell, I then wished to determine whether both T cells and non-T cells were involved in migration and whether individual cell populations would exhibit inhibition. The results described here are migration assays performed with lymph node T cells purified by nylon wool, or with non T cells obtained by treatment of lymph node cells with αThy 1.2 + C', 3 weeks after immunisation with 100 μg OVA in CFA.

Fig. 8.5 indicates that purified T cells showed migration inhibition in the presence of 0.1 and 1 mg/ml OVA which was identical to that of the whole cell population. When non T cells were examined in the assay however, conflicting results were obtained. Although overall, these cells showed significant inhibition with both 0.1 mg/ml OVA (M.I. = 0.72 $\pm$ 0.17) and 1 mg/ml OVA (M.I. = 0.80 $\pm$ 0.17), the results varied from excellent inhibition (M.I. = 0.60) to complete absence of inhibition (M.I. = 0.99) in individual tests. These variable results could be explained if T cells had been incompletely eliminated from the cell suspensions used. That this was indeed the case was confirmed by separate cytotoxicity assays in which killing of BALB/c thymocytes by αThy 1.2 + C' varied

from 70-98% (Table 8.2) in individual tests.

Conclusions

These experiments show that migration of lymph node cells in vitro was consistently inhibited in the presence of the appropriate antigen. Inhibition developed slowly after immunisation with OVA in CFA, was long-lasting and was optimal when 0.1 mg/ml OVA was used in the test system. Migration inhibition was shown to be antigen-specific and the absence of non-specific inhibition by OVA gives the test a high degree of sensitivity for this antigen. Although not absolutely clear-cut, the results from different immunisation schedules show that migration inhibition of lymph node cells is an in vitro correlate of systemic DTH as measured by skin-testing in vivo. Thus, mice immunised with 10 µg OVA in CFA have migration inhibition in the absence of serum antibodies while immunisation with OVA in IFA produces high titres of antibody but no migration inhibition and minimal DTH. However, the continuing development of migration inhibition as systemic DTH declines suggests that the in vitro and in vivo tests are not assaying identical aspects of DTH.

The majority of migrating cells were shown to be lymphocytes and it is clear that nylon wool purified T cells showed identical migration inhibition to whole lymph node cells. However, both B and T cells were found to migrate in the assay and the results of assays using non-T cell populations were difficult to interpret. The results are compatible with a residual T cell population being required for migration inhibition, and this study indicates that lymph node lymphocyte migration inhibition provides a sensitive in vitro method of assessing CMI in mouse lymphoid organs.

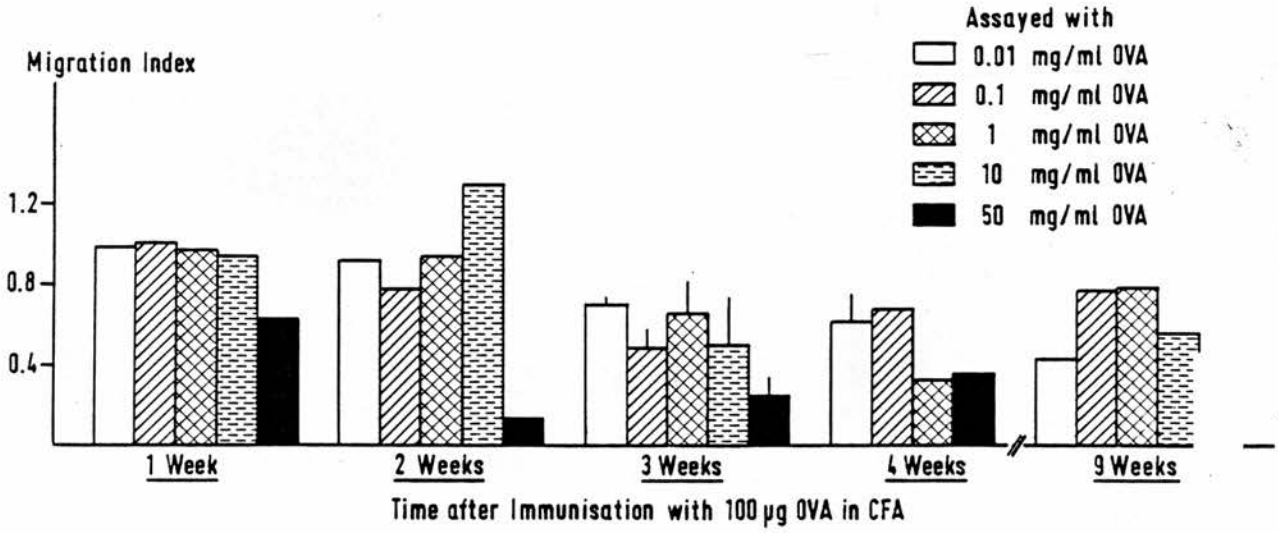

Fig. 8.1. Development of migration inhibition in the presence of different concentrations of OVA in draining lymph nodes of mice immunised with 100 µg OVA in CFA injected into the footpads. Where shown, bars represent mean Migration Index + 1 s.d. of 4 experiments. In all experiments, lymph node cells were pooled from 2-3 mice and at least 6 wells assayed for each concentration of OVA.

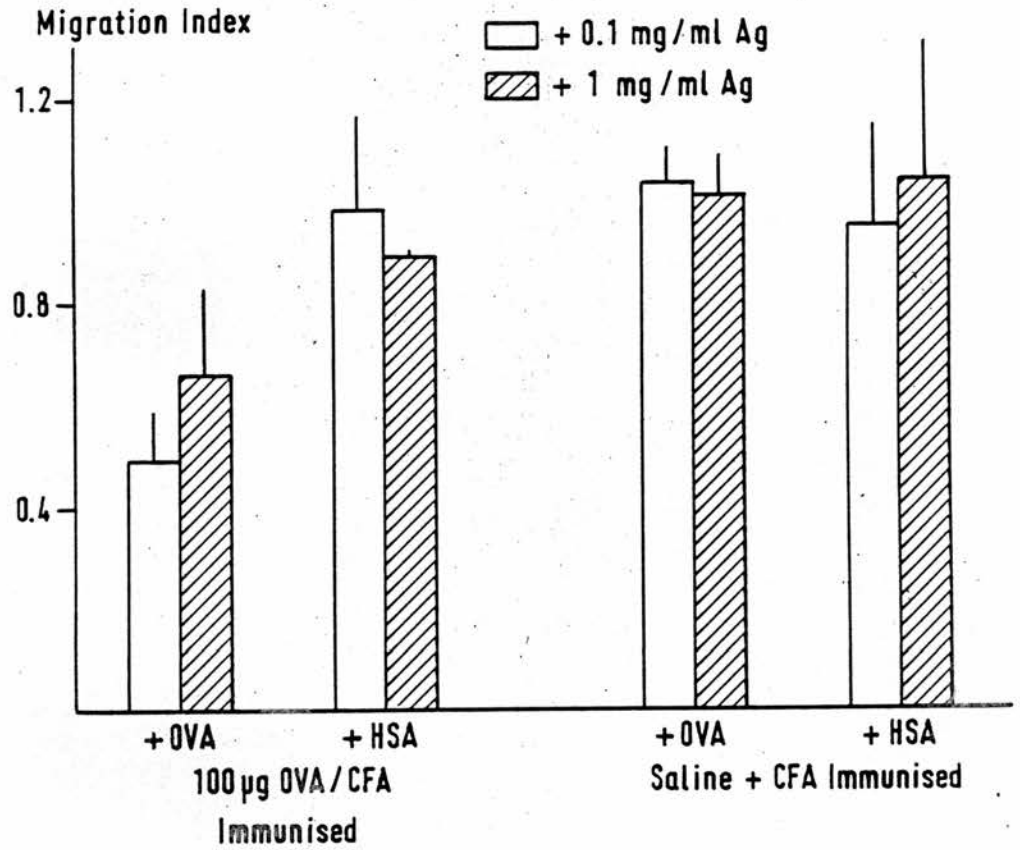

Fig. 8.2. Specificity of migration inhibition in draining lymph nodes of mice immunised with 100 µg OVA or with saline in CFA. Significant migration inhibition was observed only when OVA/CFA immunised lymph nodes were tested with 0.1 mg and 1 mg/ml OVA ($p < 0.001$ and $p < 0.005$ respectively). Each bar represents mean + 1 s.d. of 2-4 experiments using cells pooled from 2-3 mice.

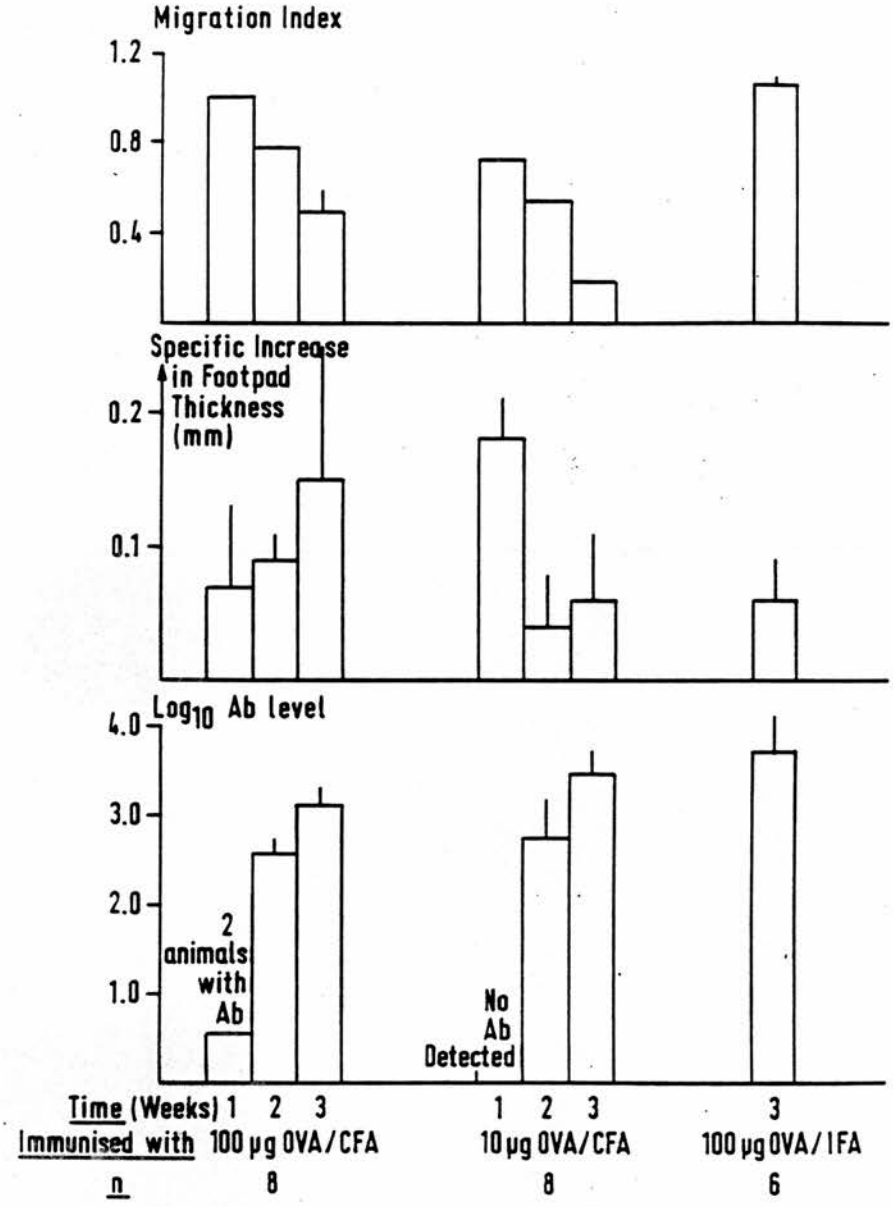

Fig. 8.3. Development of migration inhibition, systemic DTH and serum antibody responses in mice immunised with 10 µg or 100 µg OVA in CFA or with 100 µg OVA in IFA. Systemic DTH was measured by the specific increment in footpad thickness 24 hours after 100 µg OVA in saline i.d. and antibody levels by passive haemagglutination. Bars represent means + 1 s.d. (6-8 mice/group).

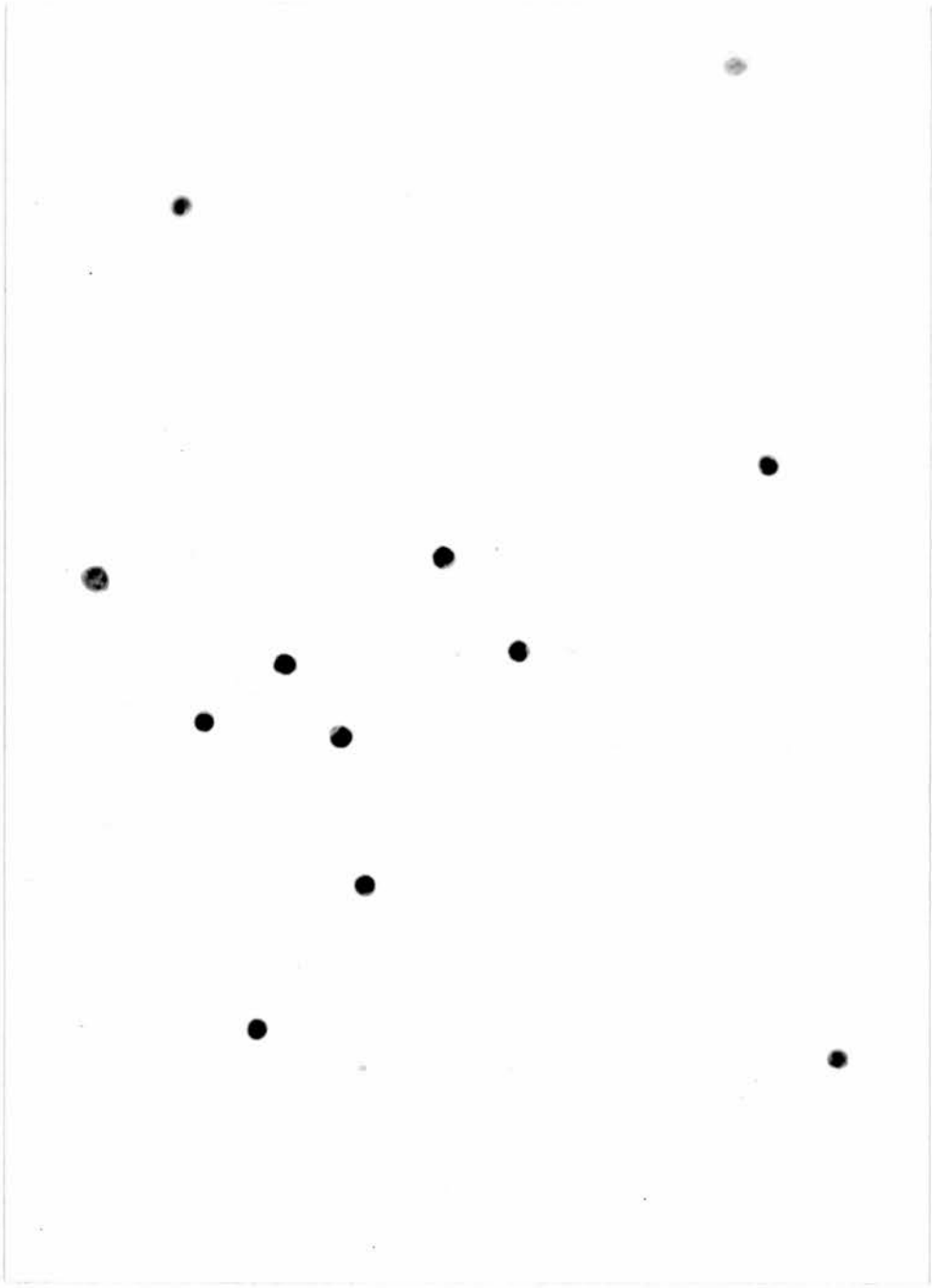

Fig. 8.4. Morphology of lymph node cells migrating from capillary tubes in vitro. The majority of migrating cells were lymphocytes. (Giemsa x 500).

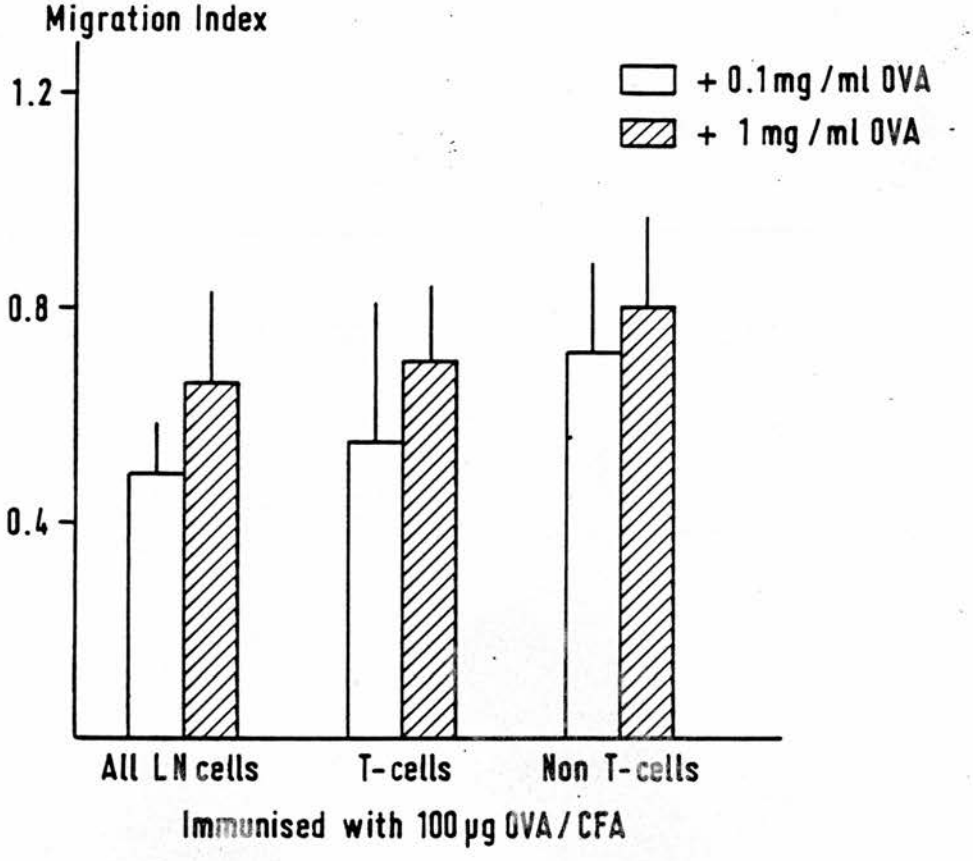

Fig. 8.5. Migration inhibition of whole lymph node cells, T cell enriched populations and non-T cells in presence of OVA 3 weeks after immunisation with 100 µg OVA in CFA. Significant inhibition of T cells was observed with both concentrations ($p < 0.02$ vs saline immunised controls in Fig. 8.2). Means + 1 s.d. of 3-4 experiments.

Lymph Node Cells		Migrating Cells
Thy 1.2 +ve	81.5% (79-86)	92% (89-94)
sIg +ve	35.5% (28-41)	37% (29-40)
T cells	46.0% (40-51)	55% (54-60)

Table 8.1. Identification of lymph node cells before and after migration in vitro as assessed by immunofluorescence. (Mean + range of 3 experiments).

	Thymus	Peripheral Lymph Nodes
% Cytotoxicity	70-98%	24-55%

Table 8.2. Ability of αThy 1.2 + C' to kill BALB/c lymphocytes in 3 experiments.

CHAPTER 9

LOCAL AND SYSTEMIC IMMUNE RESPONSES IN MICE IMMUNISED ORALLY WITH OVALBUMIN

Introduction

Although the graft-versus-host reactions discussed in Chapters 6 and 7 provide useful models of intestinal CMI, such reactions are not normally encountered during life whereas the intestinal mucosa is constantly exposed to a range of dietary proteins. My aim was to investigate the possibility that local CMI responses could be induced to a newly introduced fed protein and that this immunity might damage the small intestinal mucosa.

Feeding proteins to animals induces both local and systemic antibody responses (Crabbé, Nash, Bazin, Eyssen & Heremans 1969; Dolezel & Bienenstock 1971a,b) while systemic CMI has also been reported, although this is a rare consequence of protein feeding (Goldberg, Kraft, Peterson & Rothberg 1971; Perrotto et al 1974). In addition, local CMI has been described after feeding hapten-protein conjugates - demonstrated by production of MIF by mucosal and MLN lymphocytes (Huntley et al 1979). However, previous feeding of a protein antigen usually suppresses subsequent CMI responses to that antigen and this is related to the induction of suppressor T cells (Miller & Hanson 1979; Challacombe & Tomasi 1980). I reasoned that the induction of suppressor cells by fed antigen could explain previous failures to induce local CMI to a dietary antigen and so designed experiments in which mice depleted of suppressor cells were immunised orally with ovalbumin.

As discussed earlier, murine suppressor T cells are sensitive to certain doses of cyclophosphamide and enhanced CMI may be found when CY is given shortly before antigen (Röllinghoff et al 1977; Gill & Liew 1978; Attallah et al 1979).

I have therefore tested the hypothesis that abrogation of gut-associated suppressor cells by CY would allow the development of active local CMI in the gut and its lymphoid tissues after oral immunisation with OVA. The presence of CMI has been tested in two ways: firstly, in this section, the induction of local immunity in the MLN was studied using the lymph node migration inhibition assay described in Chapter 8. In Chapter 10, CMI in the mucosa itself was investigated using the CCPR and IEL count as indices of mucosal immunity.

In addition to studies on the induction of local immunity in orally immunised mice, the systemic CMI and antibody responses of these animals have also been assessed. This has allowed me to investigate the possibility that the presence of a local immune response in the gut need not also involve systemic immunity.

Experiments and Results

In the first series of experiments, local CMI was investigated in the MLN of mice immunised and challenged orally with OVA. Female BALB/c mice were fed 2 mg OVA by intragastric tube and 28 days later were commenced on 0.1 mg OVA/day/mouse in their drinking water. After 10 days of challenge, mice were sacrificed and the MLN used

in migration inhibition tests. In addition, one group of OVA fed mice received 100 mg/kg CY 2 days before the first feed of OVA, while a further group were given CY alone as controls (Fig. 9.1). Migration inhibition assays were also performed on the MLN from three similar groups of mice which were immunised once with 2 mg OVA and sacrificed at intervals thereafter. In studies of systemic immunity, additional groups of mice were subjected to the same protocols and tested for CMI^{I} and antibody levels. In each experiment employing MLN cells, lymph nodes from 3-4 mice from each group were pooled.

Local CMI^{I} in the MLN of mice immunised and challenged orally with ovalbumin

MLN cells from the groups described above were cultured in the presence of 1 mg/ml OVA (Fig. 9.2). There was no inhibition of migration with either MLN cells from mice fed OVA alone (M.I. = 1.00 ± 0.06) or given CY alone (1.12 ± 0.13). However, there was significant inhibition of migration of MLN cells from mice pretreated with CY and fed OVA when these mice were subsequently challenged orally with OVA (M.I. = 0.63 ± 0.18 $p < 0.01$).

Induction of local CMI^{I} in the MLN of CY treated mice after a single feed of ovalbumin

The results above indicated that CY pre-treated, OVA fed mice developed local CMI^{I} after a secondary challenge with OVA, and also confirmed that the migration inhibition assay could be applied to the MLN. I therefore decided to investigate whether a similar state of CMI^{I} would develop after a single

feed of OVA to CY treated mice and to study its time of onset and duration.

Figure 9.3 shows that significant inhibition of migration in the presence of 0.1 mg/ml OVA was apparent within 24 hours of feeding OVA and that a similar level of sensitisation persisted until at least 14 days after feeding (M.I. $\doteqdot$ 0.65). Sensitisation was then lost, but as the previous experiments showed, this could be recalled by a period of secondary oral challenge. Migration inhibition was never demonstrable in the MLN of mice fed OVA alone or given CY alone and these studies confirm the need for combining oral immunisation with CY treatment for the demonstration of local CMI.

Finally, migration inhibition of MLN cells from OVA immunised mice did not occur in the presence of HSA, nor did OVA itself inhibit migration of MLN cells from un-immunised controls (Table 9.1).

Systemic CMI responses in mice immunised and challenged orally with ovalbumin

The presence of systemic CMI was investigated either 5 or 21 days after a single feed of OVA or on completion of the 10 day oral challenge programme. CMI was assayed by the increase in double skinfold thickness 24 hours after 100 µg OVA intradermally.

A group of mice were immunised with 100 µg OVA in CFA intradermally as positive controls in these studies and when skin tested 21 days later, these mice had excellent DTH responses as measured by skin-testing (Fig. 9.4). However, no DTH could be elicited in any of the groups of orally

immunised mice. In particular, CY treated OVA fed mice had no systemic DTH either 5 days after oral priming nor on completion of the challenge protocol, despite the presence of local CM.I in the GALT at these times.

Serum antibody responses in mice immunised and challenged orally with ovalbumin

The three groups of OVA-immunised mice were also tested for the presence of haemagglutinating anti-OVA antibodies on completion of the 10 day challenge period (Table 9.2). None of these mice had detectable serum antibody at this time, while sera from mice immunised parenterally with OVA had excellent antibody levels when tested in parallel.

Conclusions

These experiments support the hypothesis that presumptive abrogation of suppressor T cells by CY will allow the development of local CM.I in the GALT following oral immunisation with a protein antigen. Thus, only mice treated with CY before initial exposure to oral OVA had OVA-dependent inhibition of migration of their MLN cells after secondary oral challenge with ovalbumin in the drinking water. Controls given CY or fed OVA alone had no evidence of CM.I in their GALT. It was also shown that sensitisation could be demonstrated in the MLN of CY treated, OVA fed mice within 1 day of a single feed of 2 mg OVA. This primary sensitisation was transient and was no longer demonstrable by 21 days after feeding. Nevertheless, it is apparent that the MLN of these mice remains primed after this time, since local CM.I

was found in the MLN of mice challenged 28 days after initial exposure to antigen. The loss of sensitisation from the MLN of mice fed a single dose of OVA may reflect emigration or differentiation of the cells required to induce migration inhibition to OVA.

In addition, the observations that mice sensitised and challenged orally with OVA can develop GALT immunity in the absence of systemic immune responses, underline the segregation of intestinal and systemic immunity.

ORAL IMMUNISATION WITH OVALBUMIN AND CYCLOPHOSPHAMIDE PRE-TREATMENT IN BALB C MICE

PROTOCOL OF IMMUNISATION

Day - 2	Cyclophosphamide 100mg/Kg I.P.
Day 1	2mg ovalbumin by intra-gastric tube
Days 28 - 37	0.1mg ovalbumin daily in drinking water
Day 37	Sacrifice

Fig. 9.1. Protocol of oral immunisation and cyclophosphamide pretreatment used to demonstrate local CMI in the GALT and intestinal mucosa.

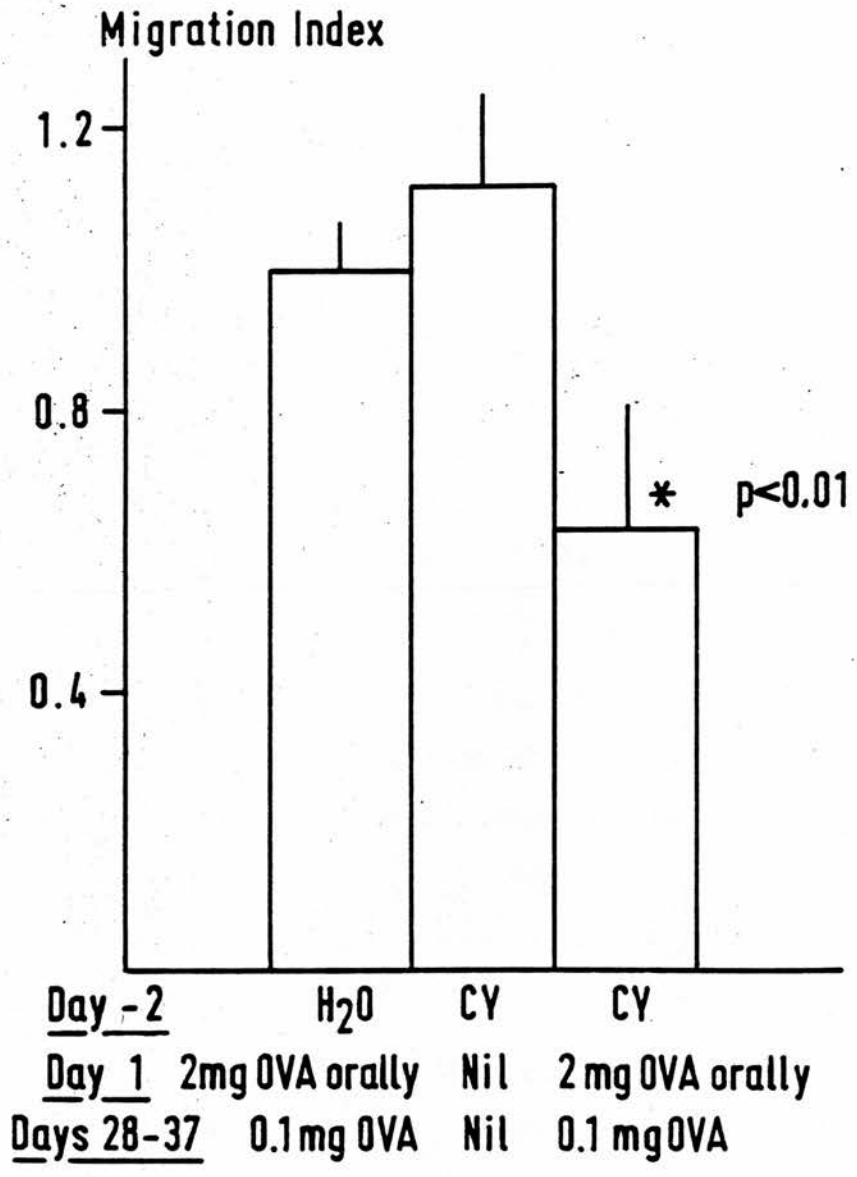

Fig. 9.2. Migration inhibition in the MLN of mice immunised and challenged orally with OVA. MLN cells from 3-4 mice were pooled in each experiment and were obtained after 10 days of oral challenge with OVA. Bars represent means + 1 s.d. of 3 experiments in which cells were assayed in presence of 1 mg/ml OVA.

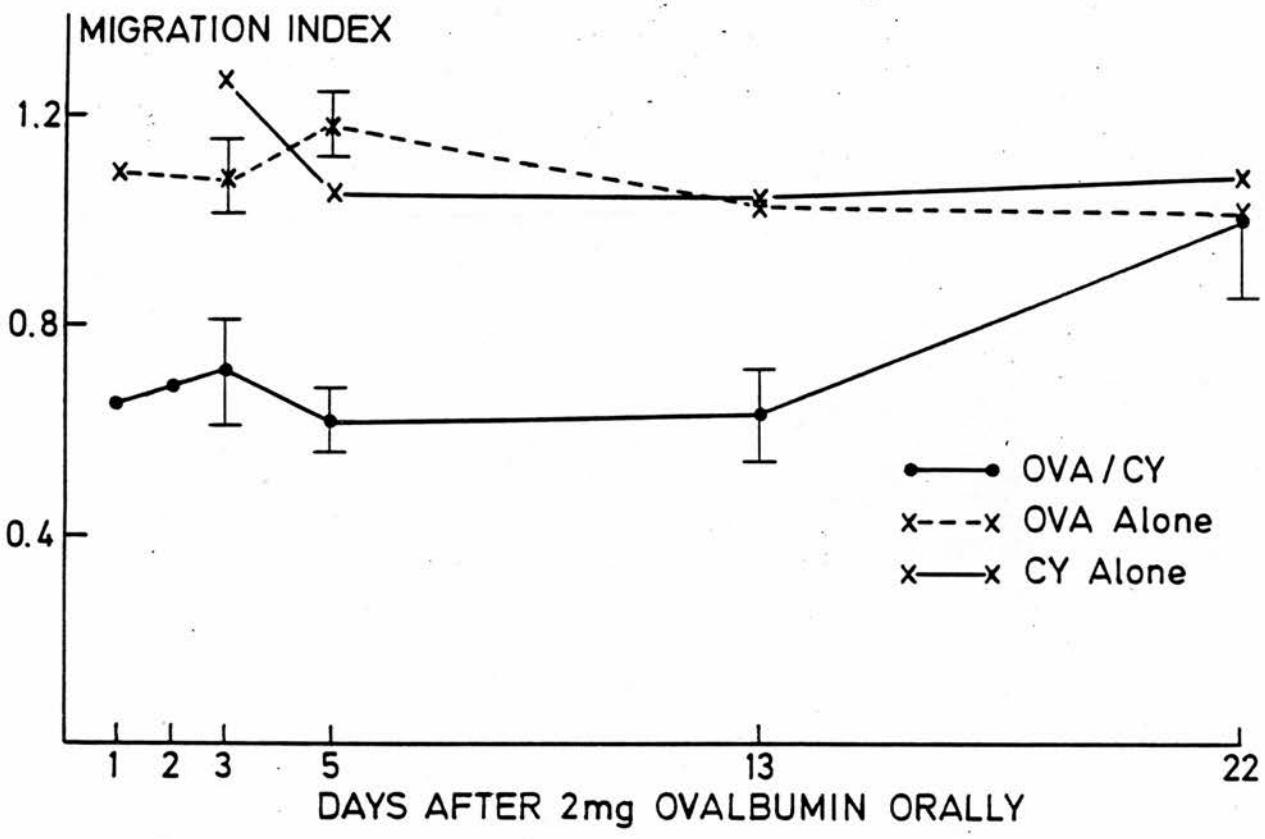

Fig. 9.3. Development of migration inhibition in the MLN of mice fed 2 mg OVA after CY pretreatment, in mice fed OVA alone, and in mice given CY alone. Cells were assayed with 0.1 mg/ml OVA and, where shown, bars represent mean $\pm$ 1 s.d. of 3 experiments (OVA/CY groups vs others $p < 0.01$). Other results are for one experiment and statistical analysis between groups is not possible. However, within each experiment at times up to 13 days, significant migration inhibition was observed in OVA/CY mice ($p < 0.02$ vs control wells).

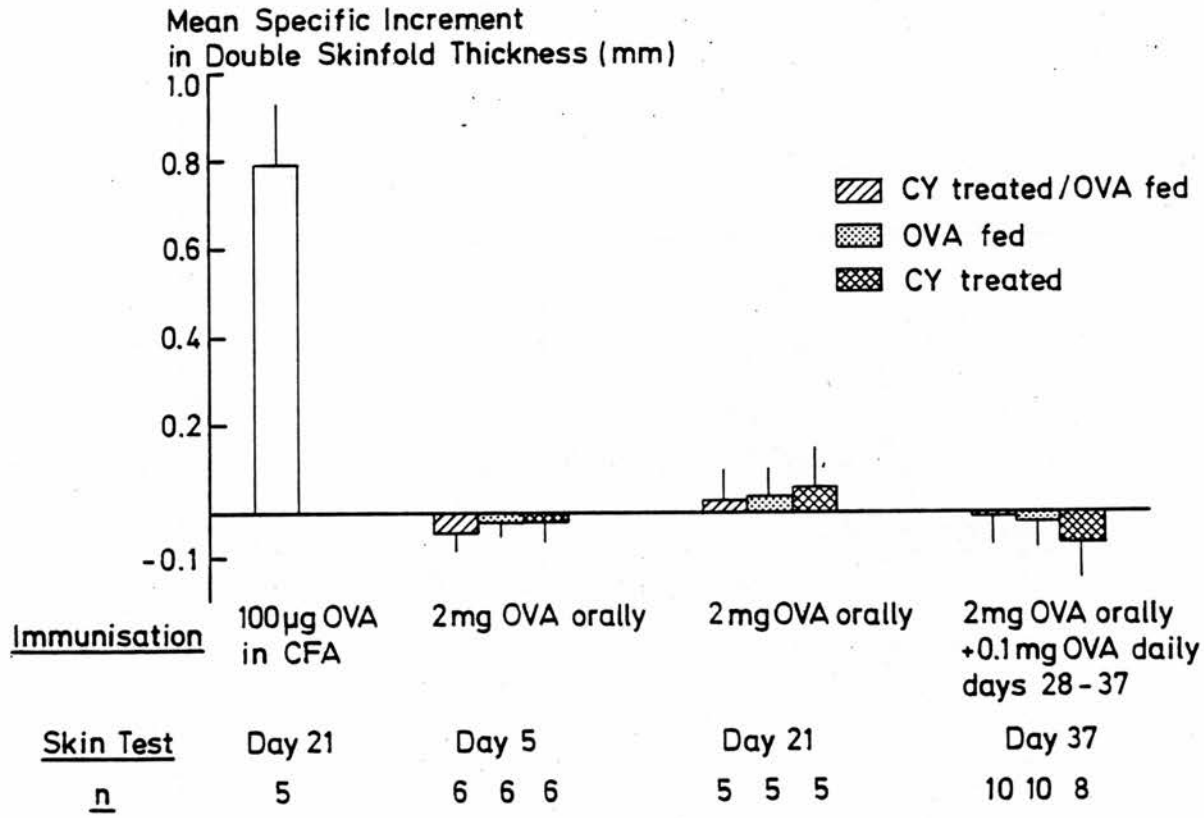

Fig. 9.4. Systemic cell mediated immunity after primary and secondary oral immunisation with OVA in mice given OVA ± CY or CY alone. A group of mice was also immunised with 100 µg OVA in CFA as positive controls. Results are mean specific increment in double skinfold thickness, 24 hours after 100 µg OVA in saline intradermally + 1 s.d.

Cell Source	No. of Experiments	Antigen	M.I.
Normal MLN	3	OVA (0.1 mg/ml)	1.01 ± 0.08
	3	OVA (1 mg/ml)	1.04 ± 0.06
MLN-OVA alone	2	HSA	0.95 ± 0.06
MLN-OVA/CY	3	HSA	0.92 ± 0.07

Table 9.1. Specificity of migration inhibition of MLN cells. Migration indices (means ± 1 standard deviation) of normal MLN cultured with OVA and of MLN from OVA immunised mice cultured with 0.1 mg/ml HSA. MLN were obtained from immunised mice 5 days after feeding OVA.

Group	n	Antibody Titre
OVA/CFA	4	4.39 ± 0.29
OVA fed	8	Not detected
CY alone	6	Not detected
OVA/CY	8	Not detected

Table 9.2. Haemagglutinating antibody responses (mean $\log_{10}$ antibody titre ± 1 standard deviation) in OVA-immunised mice. OVA/CFA mice received 2 mg OVA in CFA intradermally followed by 1 mg OVA in saline 30 days later and were bled 1 week afterwards. See text for details of orally immunised mice.

CHAPTER 10

INDUCTION OF CMI IN THE MUCOSA BY FEEDING OVALBUMIN TO CYCLOPHOSPHAMIDE TREATED MICE

Introduction

In the previous chapter, a regime of oral immunisation with ovalbumin, combined with cyclophosphamide pretreatment which led to the induction of active local immunity in the GALT was described. Since activated T cells migrate from the MLN to the intestinal mucosa (Guy-Grand et al 1974, 1978; Rose et al 1976) it is likely that cell mediated immunity in the MLN will extend to the mucosa itself. The aim of the experiments in this section was to investigate the possibility that a mucosal CMI response could be induced to the dietary protein and that mucosal damage would result from this local immunity.

I have therefore monitored the development of local CMI in the mucosa of mice immunised and challenged orally with OVA following CY treatment. The CCPR and IEL count were used as indices of mucosal CMI since these had proved reliable parameters of local immunity during the GvHR. Examination of the small intestine by microdissection should also identify any morphological abnormalities following antigen-feeding.

In addition, feeding contact sensitising agents has been reported to induce mucosal damage (Bicks et al 1967b). However, oral tolerance is the normal result of feeding these agents (Chase 1946; Glaister 1973b; Asherson et al 1977) and this unresponsiveness may be blocked by CY (Polak, Geleick & Turk 1975). In order to reinforce the studies using a protein antigen, I have also conducted a pilot study to investigate the mucosal changes after feeding oxazolone

to CY pretreated mice.

Experiments and Results

The basic outline of the experiments on OVA immunised mice is described previously (Fig. 9.1). Thus, mice were fed OVA by intragastric tube and were challenged 28 days later with OVA in the drinking water for a period of 10 days. Additional groups were given 100 mg/kg CY 2 days before the initial feed or were given CY alone as controls. In all experiments, female BALB/c mice were used and tissues were removed for examination on completion of the 10 day challenge programme.

Mucosal changes in mice immunised and challenged orally with OVA after CY treatment

In the first experiments, mice were sensitised with 2 mg OVA and received the equivalent of 0.1 mg/mouse/day as a challenge, as described in Chapter 9.

On completion of the challenge protocol, histological examination revealed no abnormalities in any of the 3 groups of animals. However, the more sensitive microdissection technique revealed significant differences between the groups (Fig. 10.1). Mice given CY alone, or sensitised and challenged alone with OVA had values for villus length, crypt length and CCPR which were similar to those of a group of untreated, normal controls included in this experiment. In mice receiving CY combined with OVA immunisation, however, the 10 day challenge resulted in significant increases in crypt length (132.4 ± 4.3 μm vs. 119.1 ± 4.7 μm, 118.4 ± 7.8 μm and 118.7 ± 5.4 μm $p < 0.001$)

and CCPR (12.0 compared to 6.6, 8.4 and 6.8). Villus lengths were similar in all groups.

Mice given CY alone or fed OVA alone had identical IEL counts to untreated controls (15.8 ± 3.0, 14.5 ± 1.2 and 14.1 ± 2.0 respectively). However, CY treated, OVA fed mice had a significant increase in IEL count after oral challenge with OVA (25.9 ± 2.9) (Fig. 10.2).

Effects on the mucosa of higher doses of oral ovalbumin

The mucosal alterations, although statistically significant, were not at all similar to the severe villus and crypt abnormalities seen in diseased patients. If mucosal changes reflect the magnitude of the mucosal immune response, higher challenge doses of ovalbumin might be expected to produce more striking alterations in the mucosa. Therefore, using the same protocol, mice were sensitised with 20 mg OVA orally and challenged orally with the equivalent of 1 mg/mouse/day for 10 days. Once again, mice given CY alone or immunised and challenged with OVA alone had similar values for villus length (802.2 ± 49 mμ and 790.9 ± 37.5 mμ), crypt length (117.7 ± 7.2 mμ and 112.1 ± 2.2 mμ) and CCPR (7.3 and 5.6), while CY treated, OVA fed animals showed a significant increase in CCPR (12.5) and crypt length (125.3 ± 5.7 mμ). In addition, these mice also had a minor, but significant increase in villus length (852.7 ± 20 mμ $p < 0.02$) (Fig. 10.3). Intraepithelial lymphocyte counts (Fig. 10.4) in CY treated and OVA fed controls were similar (17.8 ± 0.9 and 16.2 ± 1.00), while mice receiving both had a significantly raised IEL count

in comparison (25.5 ± 3.5). Once again, there were no histological abnormalities in these animals.

The tenfold increase in dose of fed antigen has thus had no significant effect on the resulting mucosal alterations in CY treated mice.

Antigen specificity of mucosal responses to fed antigen

It was important to ensure that the changes described did not merely represent a non-specific response of the CY treated mucosa to dietary proteins. Mice were therefore fed 2 mg BSA on day 1 and challenged as before with 0.1 mg/day OVA in the water. Again, one group of BSA fed mice received 100 mg/kg CY 2 days before feeding and another group received CY alone. As shown in Fig. 10.5, all 3 groups of mice had similar values for all indices of mucosal architecture. Mice receiving CY before oral immunisation with BSA and challenge with OVA had no increase in crypt length (100.6 ± 2.7 mμ) or CCPR when compared to CY treated or BSA/OVA fed controls which had similar values for crypt length (99.7 ± 2.8 mμ and 99.6 ± 2.8 mμ) and CCPR. In addition, all three groups had identical IEL counts (Fig. 10.6).

Thus, the alterations in mucosal architecture and in lymphocytic infiltration found after oral immunisation and challenge of CY treated mice are specific to the antigen used to first immunise the animal.

Transfer of syngeneic thymocytes to CY treated, orally immunised mice

In order to investigate the possibility that the observed effects of CY were due to its action on suppressor

cells, I decided to perform a preliminary study on the effects of reconstituting CY treated mice with normal thymocytes.

Mice were immunised with 2 mg OVA orally ± CY treatment and challenged with 0.1 mg/day OVA as usual. In this experiment, one additional group of CY treated, OVA fed mice received 4×10^7 syngeneic BALB/c thymocytes i.p. immediately before primary immunisation. Once again, CY treated and OVA fed controls had similar values for villus length (763.2 ± 48.2 mμ and 693.1 ± 49.0 mμ), crypt length (103.4 ± 2.5 mμ and 103.3 ± 2.9 mμ) and CCPR (3.4 and 3.7) while CY treated, OVA fed mice had normal villus lengths but significant increases in crypt length (116.6 ± 4.9 mμ) and CCPR (10.3) (Fig. 10.7). Similarly, Fig. 10.8 shows that CY treated and OVA fed controls had identical IEL counts (13.8 ± 0.6 and 13.7 ± 1.3) while mice receiving both had a significant elevation of their IEL count (22.7 ± 2.9). These results therefore confirm the findings of the previous experiment. In addition, transfer of syngeneic thymocytes did not prevent the mucosal alterations induced by CY treatment and oral immunisation and challenge with OVA. These mice had villus lengths similar to all other groups, but had significant increases in crypt length (114.6 ± 3.0 mμ), CCPR (10.1) and IEL count (23.0 ± 1.9). These values were identical to those obtained in the CY treated, OVA fed group.

Pilot study on feeding oxazolone to CY treated mice

In this study, mice were fed 10 mg oxazolone in 0.3 ml olive oil followed by a second feed of 2 mg oxazolone in

olive oil 7 days later. The mice were sacrificed 2 days after the challenge dose. One group of oxazolone-fed mice also received 100 mg/kg CY 2 days before the first feed. It can be seen from Table 10.1 that mice fed oxazolone and given CY had a significant increase in crypt length and a large increase in CCPR compared to oxazolone-fed controls (9.5 vs. 2.7). In addition, CY treated, oxazolone-fed mice had a significantly raised IEL count compared to their controls.

The results from this pilot study with small numbers of mice appear to confirm the ability of CY to induce mucosal changes consistent with local CMI following oral immunisation and challenge with a non-protein antigen.

Conclusions

These experiments support the concept that treatment of mice with 100 mg/kg CY 2 days before primary oral immunisation with antigen, allows the development of specific alterations in the intestinal mucosa on subsequent oral challenge with antigen. The mucosal changes observed included significant increases in CCPR, crypt length and IEL count and were therefore similar to those found during the intestinal phase of the GvHR. These changes were found only on challenge with the antigen used to immunise the animals and it is probable that they represent the induction of a mucosal CMI response to a dietary antigen. This is supported by the results of the previous chapter which demonstrated the presence of local CMI in the GALT of CY treated mice immunised and challenged orally with OVA.

The mucosal abnormalities observed were not severe and were not increased by the use of larger doses of fed antigen. It is possible that more prolonged exposure of the mucosa to antigen will be required for the induction of more severe intestinal damage, such as villous atrophy. Although depletion of suppressor cells is the presumed mechanism for the action of CY, transfer of normal thymocytes did not prevent the mucosal changes in CY treated, OVA fed mice. In these experiments, thymocytes were transferred at the same time as the initial feed of OVA and are therefore unlikely to have been present in the intestine to encounter the fed antigen. It is therefore probable that this transfer did not reconstitute the animals with functioning suppressor cells in the gut at the time of feeding.

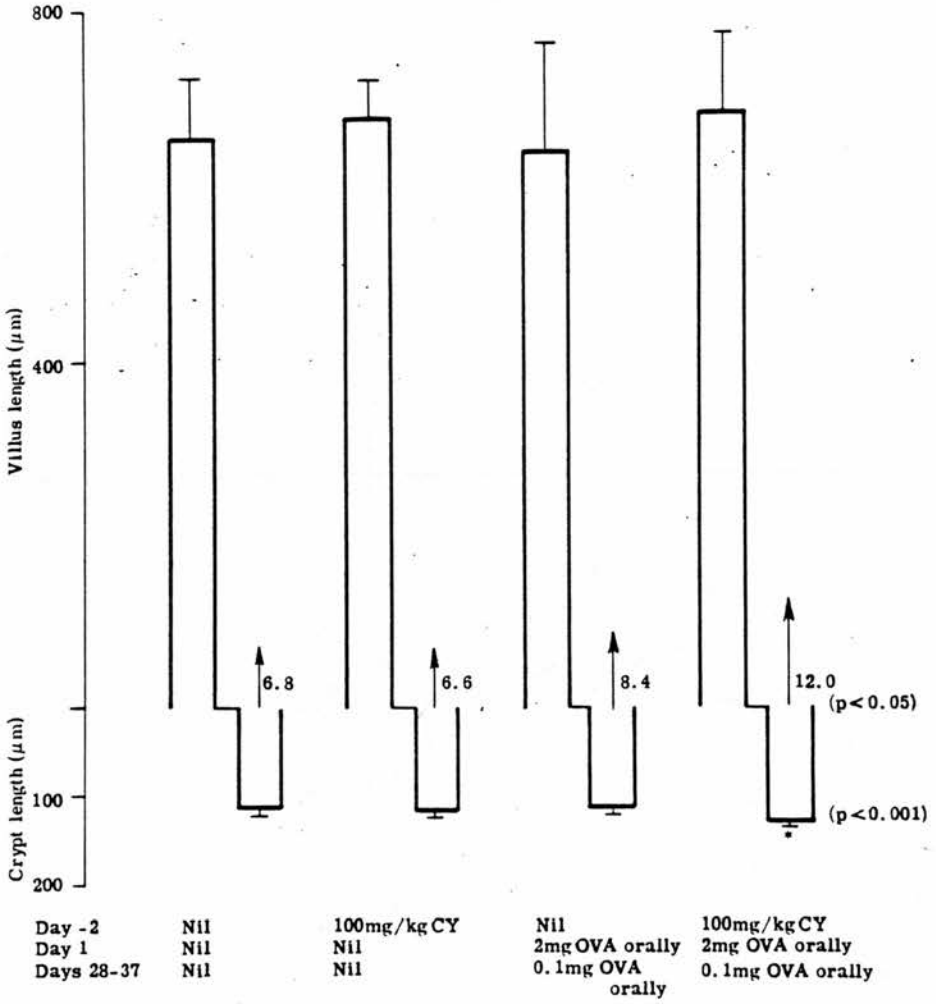

Fig. 10.1. Mucosal architecture in jejunum of BALB/c mice fed 2 mg OVA after CY pretreatment, in mice given OVA or CY alone, and in normal controls. Mucosal changes measured after 10 days of oral challenge with 0.1 mg OVA/day. Bars show means + 1 s.d. for villus/crypt length and arrows represent CCPR (6-8 mice/group).

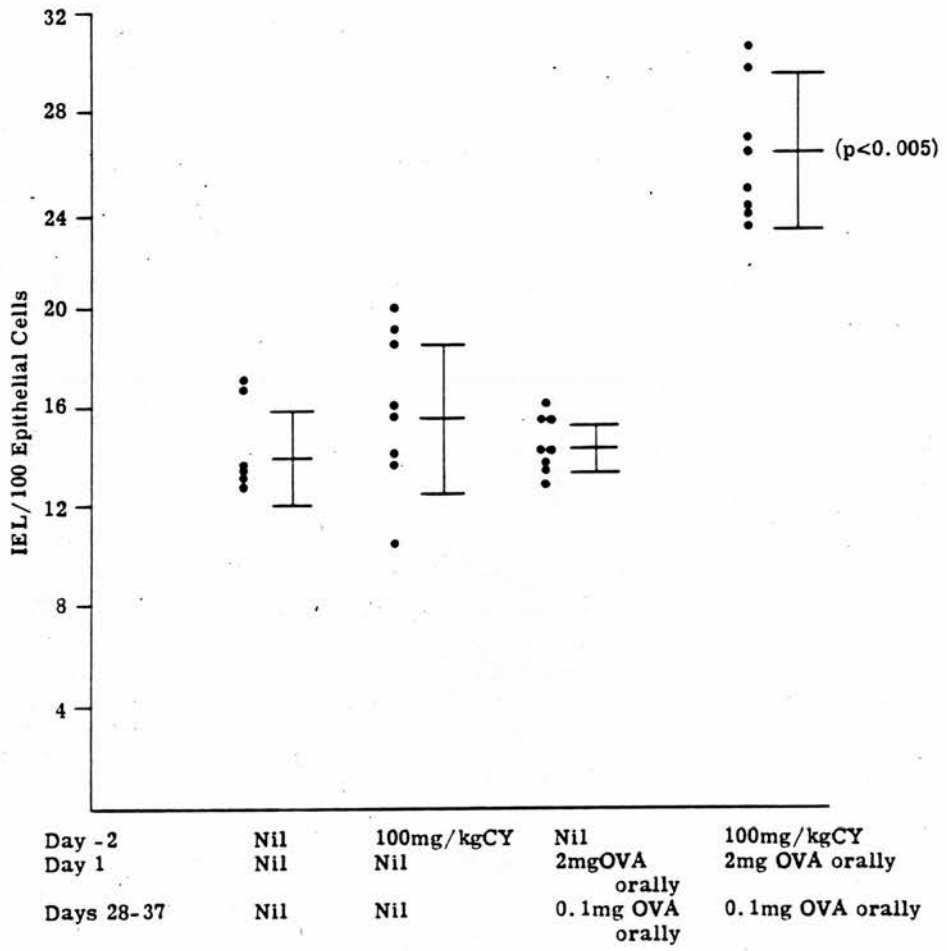

Fig. 10.2. Intraepithelial lymphocyte counts in jejunum of BALB/c mice fed 2 mg OVA after CY pretreatment, in mice given OVA or CY alone, and in normal controls. IEL counts measured after 10 days of oral challenge with 0.1 mg OVA/day. Results are means $\pm$ 1 s.d. for each group of 6-8 mice.

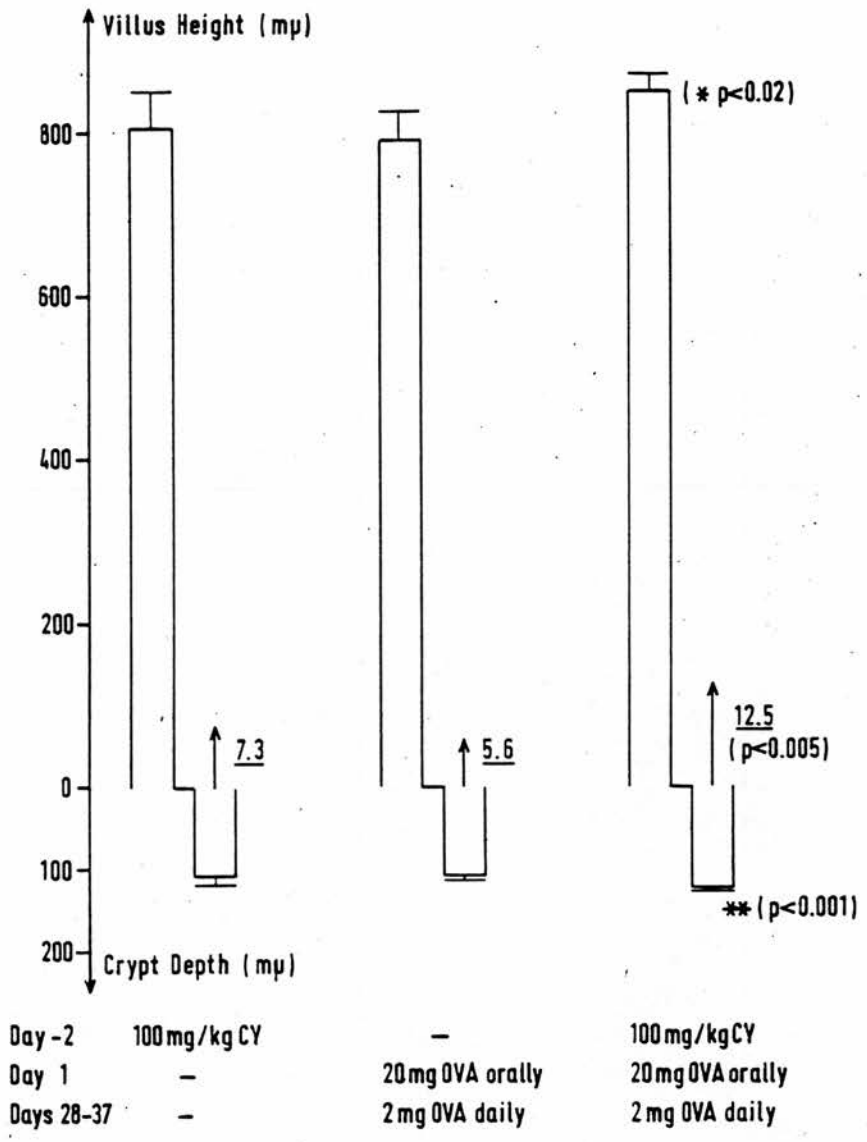

Fig. 10.3. Effect of feeding larger doses of OVA on the induction of mucosal CMI in BALB/c mice. Mucosal architecture in jejunum of CY pretreated mice fed 20 mg OVA and challenged with 1 mg OVA/day orally for 10 days and in mice given CY or OVA alone. Bars represent means + 1 s.d. for villus/crypt length and arrows show CCPR (6-8 mice/group).

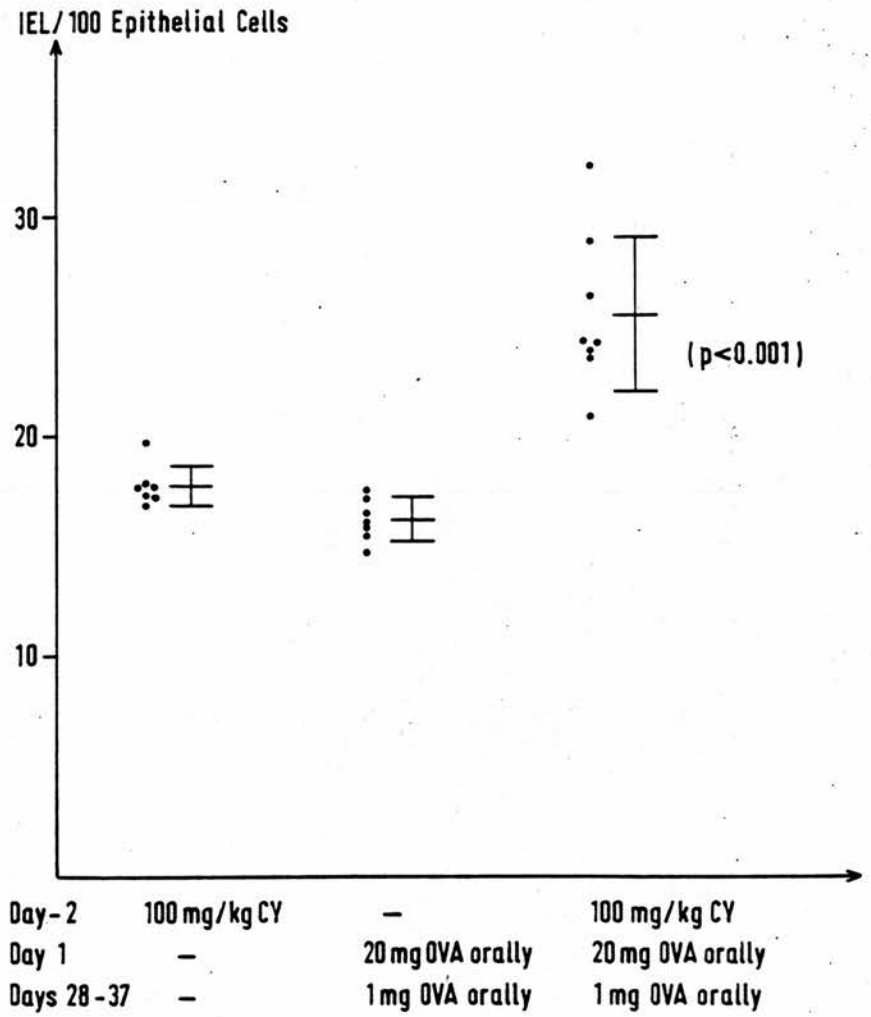

Fig. 10.4. Effect of feeding larger doses of OVA on the induction of mucosal CMI in BALB/c mice. Intraepithelial lymphocyte counts in jejunum of CY pretreated mice fed 20 mg OVA and challenged with 1 mg OVA/day orally for 10 days and in mice given CY or OVA alone. Bars represent means ± 1 s.d. for groups of 6-8 mice.

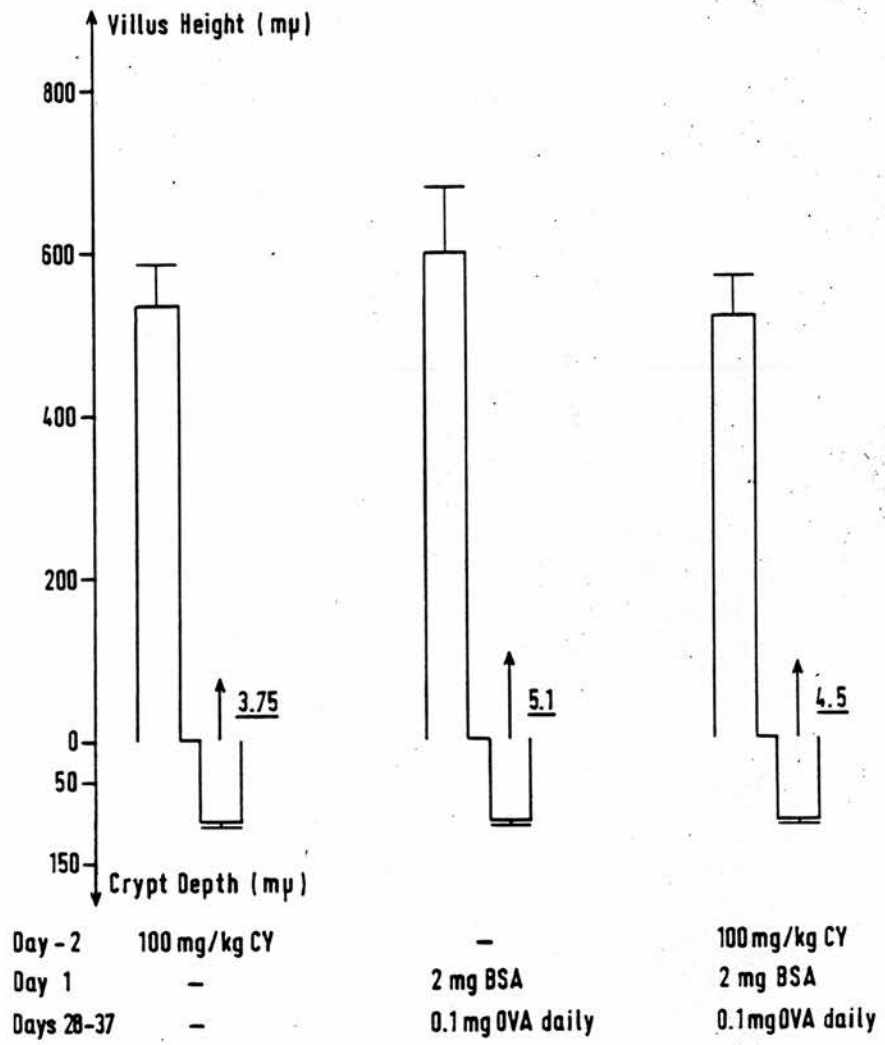

Fig. 10.5. Specificity of mucosal CMI to fed OVA. Mucosal architecture in CY pretreated mice fed 2 mg BSA and challenged orally with 0.1 mg OVA/day and in mice given CY alone or fed BSA and OVA. Bars represent means + 1 s.d. for villus/crypt length and arrows represent CCPR. No significant changes were observed (7-8 mice/group).

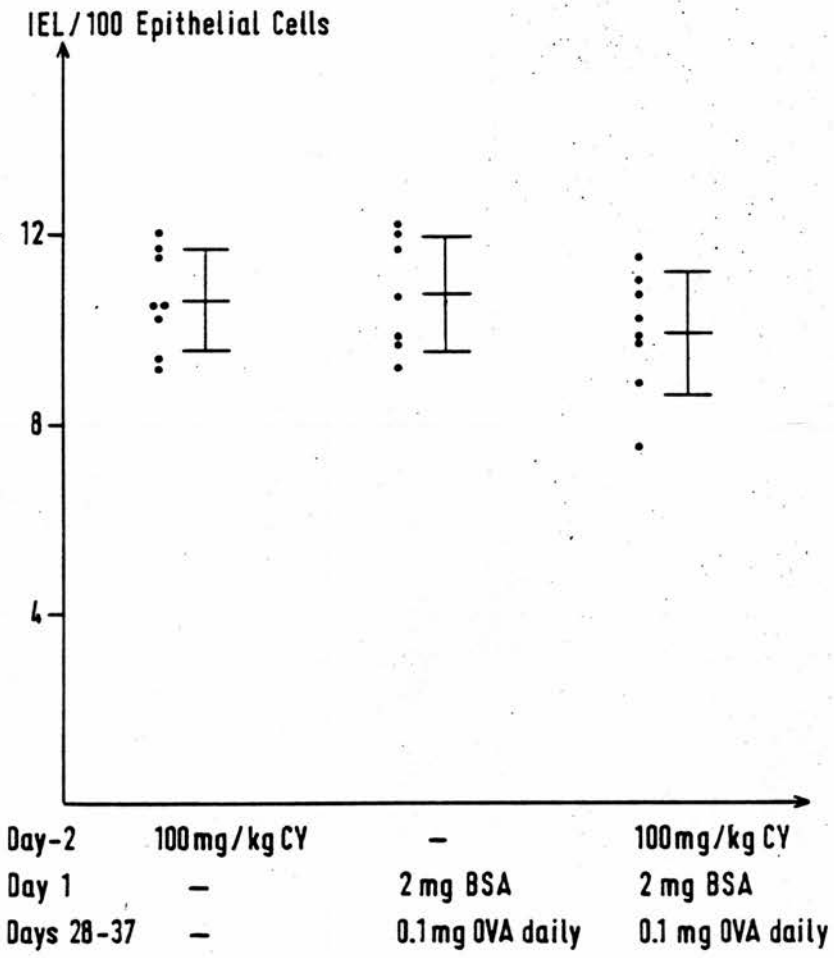

Fig. 10.6. Specificity of mucosal CMI to fed OVA. Intra-epithelial lymphocyte counts in CY pretreated mice fed 2 mg BSA and challenged orally with 0.1 mg OVA/day and in controls. Bars show means ± 1 s.d. for each group of 7-8 mice. No significant differences were observed.

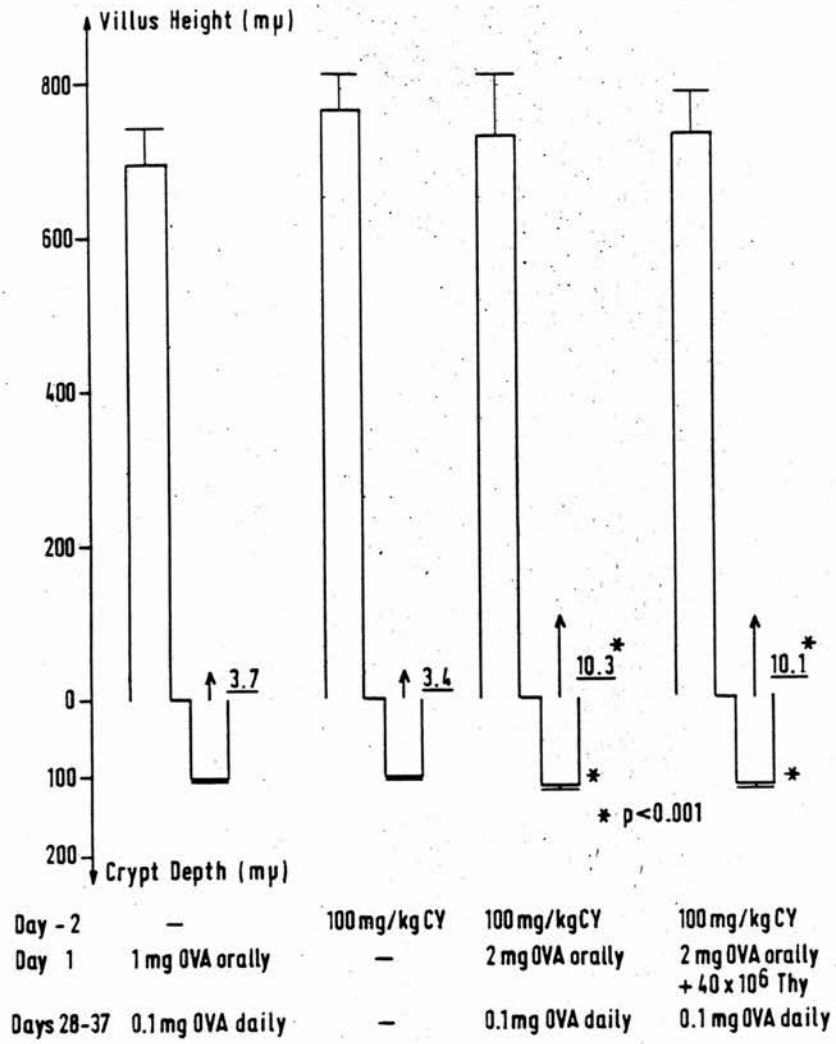

Fig. 10.7. Effect of syngeneic thymocytes on the induction of mucosal CMI to fed OVA. Mucosal architecture in BALB/c mice pretreated with CY and injected i.p. with 4×10^7 normal thymocytes before feeding 2 mg OVA and oral challenge with 0.1 mg OVA/day. Controls received CY + OVA, or were given CY or OVA alone. Bars represent means + 1 s.d. for villus/crypt length and arrows show CCPR for each group of 5-8 mice.

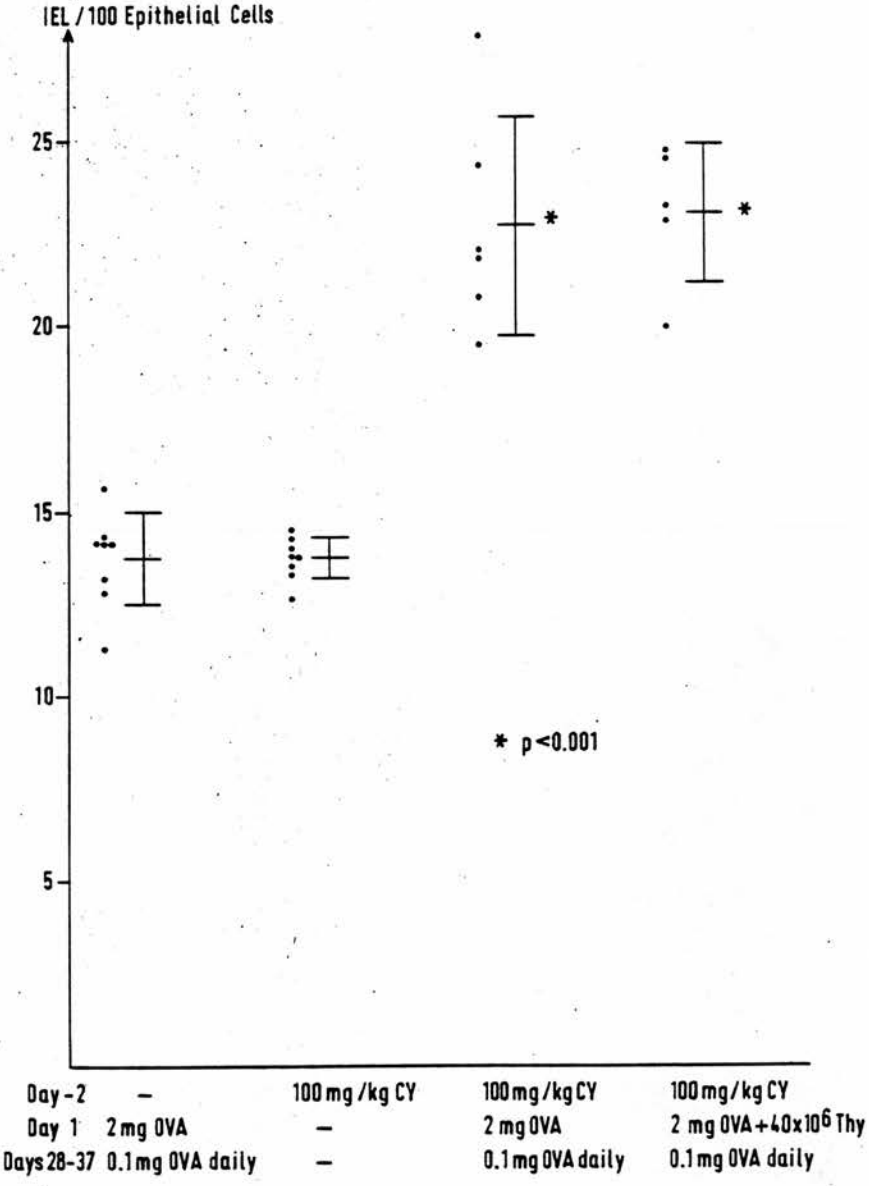

Fig. 10.8. Effect of syngeneic thymocytes on the induction of mucosal CMI to fed OVA. Intraepithelial lymphocyte counts in BALB/c mice pretreated with CY and injected i.p. with 4×10^7 normal thymocytes before feeding 2 mg OVA and oral challenge with 0.1 mg OVA/day. Controls received CY + OVA or were given CY or OVA alone. Bars represent mean $\pm$ 1 s.d. for each group of 5-8 mice.

Group	n	Villus Length	Crypt Length	CCPR	IEL
OXAZ	4	601.8 ± 66.6 mμ	104.5 ± 4.2 mμ	2.7	7.6 ± 1.5
OXAZ/CY	4	598.6 ± 40.0 mμ	126.4 + 5.3 mμ*	9.5**	14.6 ± 2.1*

Table 10.1. Mucosal changes in mice fed and challenged orally with oxazolone after CY treatment. (means ± 1 standard deviation).

See text for details of oral immunisation schedule

(* $p < 0.005$)
(** $0.05 < 0.10$)

CHAPTER 11

ABROGATION OF ORAL TOLERANCE TO OVALBUMIN BY CYCLOPHOSPHAMIDE

Introduction

Within the spectrum of immunological responses to fed antigen, systemic unresponsiveness is more often encountered than active immunity (see Chapter 2). In the preceding Chapters, however, I found that cyclophosphamide allowed the induction of active, local CMI in the GALT and intestinal mucosa of mice immunised orally with ovalbumin.

In these experiments, the consequences of CY treatment were considered to be secondary to its inhibitory action on suppressor lymphocytes. Since suppressor T cells have been implicated in the induction of oral tolerance by feeding OVA (Richman, Chiller, Brown, Hanson & Vaz 1978; Ngan & Kind 1978; Miller & Hanson 1979), I wished to investigate whether CY would also interfere with oral tolerance to OVA as well as allowing local priming for CMI. The first objective of the experiments described here was thus to study the effect of feeding OVA to CY treated mice on the subsequent systemic immune response.

Systemic CMI and humoral immunity may differ in their susceptibility to tolerance induction by intravenous antigen and may therefore be subject to different regulatory mechanisms in the unresponsive animal (Neveu & Borduas 1974; Silver and Benacerraf 1974). It has also been proposed that several regulatory factors, including suppressor cells, may be responsible for oral tolerance after feeding OVA (Hanson, Vaz, Maia & Lynch 1979). The second objective of these experiments was therefore to investigate whether

systemic CMI and humoral immunity would differ in their susceptibility to tolerance induction by feeding OVA. In this way, I hoped to find evidence for the regulation of these responses by different mechanisms and to clarify the role of suppressor cells in the regulation of CMI. Therefore, in these experiments, CY treated mice were fed two different doses of OVA and their systemic CMI and antibody responses measured after subsequent parenteral immunisation with OVA.

Experiments and Results

Protocol of oral tolerance induction and cyclophosphamide pre-treatment

This is shown in Fig. 11.1. Mice in Group 1 were controls immunised with 2 mg OVA in CFA i.p. and bled weekly for antibody levels or with 100 μg OVA in CFA intradermally and tested for DTH by intradermal footpad test 3 weeks later. Group 2 mice were fed either 2 mg or 25 mg OVA as tolerising procedure 2 weeks before immunisation, while Group 3 mice received 100 mg/kg CY 2 days before feeding OVA. Where appropriate, results in Group 2 and 3 are expressed as % suppression of control values given as:

$$\% \text{ Suppression} = \left(\frac{\text{Control Value} - \text{Value in Fed Mice}}{\text{Control Value}}\right) \times 100\%$$

Effects of OVA prefeeding on serum antibody responses to parenteral OVA

1. IgM levels. Figs. 11.2 and 11.3 show the mercaptoethanol-sensitive antibody responses in mice immunised intraperitoneally with 2 mg OVA in CFA, two weeks after feeding 2 mg or 25 mg OVA respectively. The peak of the IgM response occurred

two weeks after immunisation in animals fed 2 mg OVA and at one week in mice fed 25 mg OVA, at which times virtually all antibody was of the IgM class. It is seen that at these times, both doses of oral antigen suppressed the subsequent IgM response compared to controls, with 93% suppression ($p < 0.01$) in 25 mg OVA fed animals (Fig. 11.3a) and 60% suppression in 2 mg OVA fed mice ($p < 0.05$) (Fig. 11.2a). The groups of mice pretreated with CY, before OVA feeding, had IgM levels which were identical to controls after feeding 25 mg OVA and were not significantly different from controls in the 2 mg OVA fed group (36% suppression).

2. IgG levels. In contrast to the results with the IgM responses in OVA fed mice where both doses of OVA suppressed the response, there was a marked discordance in the effect of 2 mg or 25 mg OVA on subsequent IgG responses (Figs. 11.2 and 11.3). Mercaptoethanol-resistant antibody accounted for the majority of the antibody three weeks after immunisation and it can be seen that at this time, feeding 25 mg OVA had markedly suppressed the IgG response of fed mice compared to controls (93% suppression $p < 0.005$) (Fig. 11.3f). Mice fed 2 mg OVA on the other hand had IgG responses which were identical to controls. CY pretreatment of mice fed 25 mg OVA returned their IgG response towards normal (77% suppression) although this was not significantly different from either tolerant or control animals. CY pretreated, 2 mg OVA fed mice, had IgG levels which were the same as both other groups in this experiment.

3. Time course of antibody responses in mice fed 25 mg OVA.

It was of interest to know if the maturation of the antibody response was also altered in the mice rendered tolerant by oral protein. In the three groups of mice fed 25 mg OVA, serum IgM and IgG levels could be followed from 1-3 weeks after immunisation and these can be seen in Figs. 11.3a-e. All groups showed the characteristic early peak of IgM production at one week (Fig. 11.3a) with higher levels of IgG later in the experiment (Figs. 11.3d-f). However, it is notable that after the marked suppression of IgM levels early in the response of the OVA fed mice, there was in addition a late rise in IgM antibody in these animals (Fig. 11.3c). This secondary rise in IgM three weeks after immunisation was mirrored by a decline in IgG at this time (Fig. 11.3f) and was in marked contrast to the responses of control animals in whom IgM levels were very low by then. CY pretreated mice, after an early IgM response similar to controls (Fig. 11.3a) had at later times a pattern of IgG and IgM responses which was midway between the control and tolerant animals (Figs. 11.3c and 11.3f). When the results at two weeks were compared, the pattern of this response in the three groups was already emerging and was clearly associated with the switch from IgM and IgG production in the controls (Figs. 11.3b and 11.3e).

Thus, the results in Figs. 11.2 and 11.3 indicate that the IgM responses of mice could be tolerised by even low doses of oral protein while larger amounts were required

to achieve suppression of the IgG response. Also the tolerance of IgM responses appeared more susceptible to abrogation by CY pretreatment. Variation between individual groups at each time in this experiment was quite large; however, the overall trend of the results in Fig. 11.3 indicates that the larger dose of oral OVA interfered with the maturation of the primary antibody response as well as suppressing absolute levels of antibody.

DTH responses in OVA fed mice

Figure 11.4 shows the DTH responses in mice fed 2 or 25 mg OVA, assessed three weeks after immunisation by measuring the specific increase in footpad thickness 24 hours after an intradermal injection of 100 µg OVA in H_2O. The results in this figure illustrate that both doses of oral OVA suppressed a subsequent DTH response, with 88% suppression ($p = 0.05$) in 25 mg OVA fed mice and 64% suppression in the mice fed 2 mg OVA ($p = 0.05$) compared to controls. CY given to mice fed 25 mg OVA returned their systemic DTH response to a value which is midway between the control and tolerant mice and which was not significantly different from either. It is important to note, however, that the mice receiving CY before feeding 2 mg OVA had completely normal DTH responses, and showed no residual tolerance. Similar results were obtained 48 hours after testing (Fig. 11.4), confirming the DTH nature of the reactions measured, and the consistency of the tolerant state. Thus, feeding 25 mg OVA suppressed the humoral IgM, IgG and CMI responses while 2 mg OVA orally

was effective only in reducing CMI and to a lesser extent the IgM antibody response. Furthermore, while both doses of oral OVA used produced significant suppression of subsequent DTH responses, the tolerance after feeding 25 mg OVA was only partially susceptible to CY pretreatment. Tolerance found after 2 mg OVA orally on the other hand was completely abrogated by CY.

Antigen specificity of oral tolerance induced by feeding OVA

To confirm that the state of tolerance induced by feeding OVA was specific to the fed protein, mice were also immunised with 2 mg HSA in CFA two weeks after feeding 25 mg OVA. Fig. 11.5 shows the haemagglutinating anti-HSA responses one and three weeks after immunisation. The OVA fed mice exhibited antibody responses to the unrelated protein which were the same as those of the H_2O fed controls.

Similarly, Figure 11.6 indicates that three weeks after intradermal immunisation with 100 µg HSA in CFA, mice fed 25 mg OVA also had normal DTH responses to HSA, assessed as the specific increase in footpad thickness 24 hours after 100 µg HSA in H_2O intradermally.

Effect of cyclophosphamide on systemic immunity

Since CY had been found to increase the systemic immune response of mice fed tolerogenic doses of OVA, it was important to exclude a residual effect of CY acting on systemic immunity at the time of parenteral immunisation.

Thus, mice were given 100 mg/kg CY 16 days before intradermal immunisation with 100 µg OVA in CFA. Serum

antibodies and systemic DTH were assessed as before, and Figures 11.7 and 11.8 show that CY pretreatment had no significant effect on these responses. In the case of the humoral response, reduction rather than enhancement of antibody levels was observed. It is therefore unlikely that the effect of CY on oral tolerance can be explained by a stimulatory action or systemic immunity.

Ovalbumin was not a constituent of the mouse diet

Since the studies in these Chapters were based on the assumption that OVA was a new dietary protein in the mice investigated, it was essential to ensure that the normal laboratory rodent diet did not contain OVA. The diet used in the Animal Unit at the Western General Hospital contained no ingredients of obvious egg origin (Spratts Ltd. - personal communication). However, trace amounts of OVA were sought by analysing both a crude extract and a salt precipitated fraction of the mouse diet. Antigenic OVA was identified using the haemagglutination inhibition assay described in Materials and Methods and this was shown to detect levels of $\geqslant$ 1 µg/ml OVA.

Neither extract of diet was found to cause haemagglutination inhibition of OVA coated SRBC, despite the fact that the extracts used were highly concentrated preparations containing the equivalent of 20 g food.

Summary and Conclusions

These experiments have shown that feeding 25 mg OVA two weeks before parenteral immunisation will reduce both humoral

and cell mediated immunity to OVA. However, when mice were fed 2 mg OVA, only CMI and IgM antibody responses were suppressed, and IgG antibody levels were unaffected. Cyclophosphamide was found to block partially the tolerance induced by feeding 25 mg OVA and to abrogate completely the induction of tolerance of CMI found after feeding 2 mg OVA.

Thus, 100 mg/kg CY 2 days before feeding OVA modulates the resulting state of systemic unresponsiveness as well as permitting the development of active CMI in the GALT and mucosa.

The results indicate that systemic CMI is readily tolerised by even low doses of fed OVA and that this tolerant state is fully sensitive to CY. This finding is compatible with the theory that a CY-sensitive suppressor cell system is normally activated by small amounts of a new dietary protein and that this mechanism is important in the phenomenon of oral tolerance. These cells may also inhibit the development of local CMI in the gut and its lymphoid tissues.

In addition, the results underline the multiple consequences of feeding protein antigens. Hence, suppression of systemic CMI and antibody responses were dependent on the dose of OVA fed and CY had differing effects on these tolerant states. These findings suggest that oral tolerance is dependent on the induction of several regulatory factors, including suppressor cells. The outcome of feeding antigen will depend on the dose and nature of antigen and the various regulatory mechanisms which are activated.

Group	Day-2	Day 1	Day 14	
1. Control	H_2O i.p.	H_2O orally	2 mg OVA/CFA i.p. or 100µg OVA/CFA i.d.	Bled weekly for Ab Tested for systemic DTH day 35
2. OVA fed	H_2O i.p.	2 mg or 25 mg OVA orally		
3. CY/OVA fed	100 mg/kg CY i.p.	2 mg or 25 mg OVA orally		

Fig. 11.1 Protocol for the induction of tolerance by feeding ovalbumin and its abrogation by cyclophosphamide.

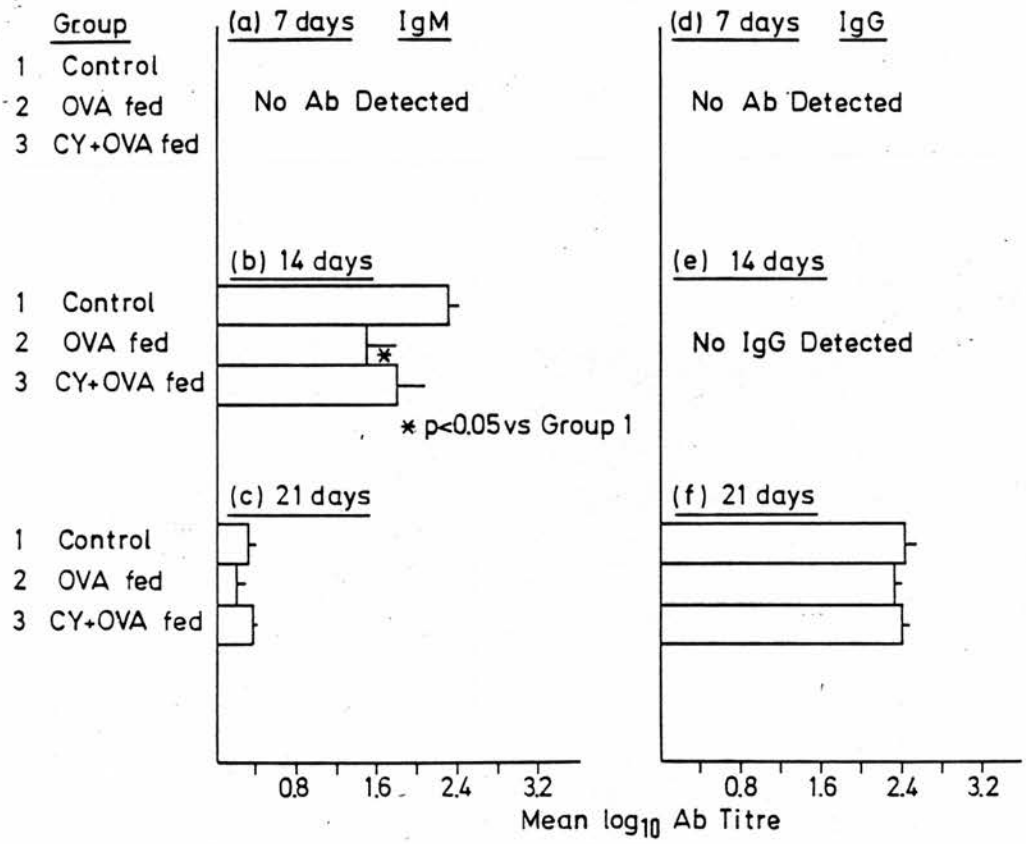

Fig. 11.2. Effect of CY on the induction of tolerance by feeding 2 mg OVA to BALB/c mice. Serum haemagglutinating IgM and IgG antibody levels 7,14 and 21 days after 2 mg OVA in CFA i.p. Mice were fed 2 mg OVA 14 days before immunisation. Bars represent mean $\log_{10}$ antibody titres + 1 s.e.m. for each group of 6-8 mice.

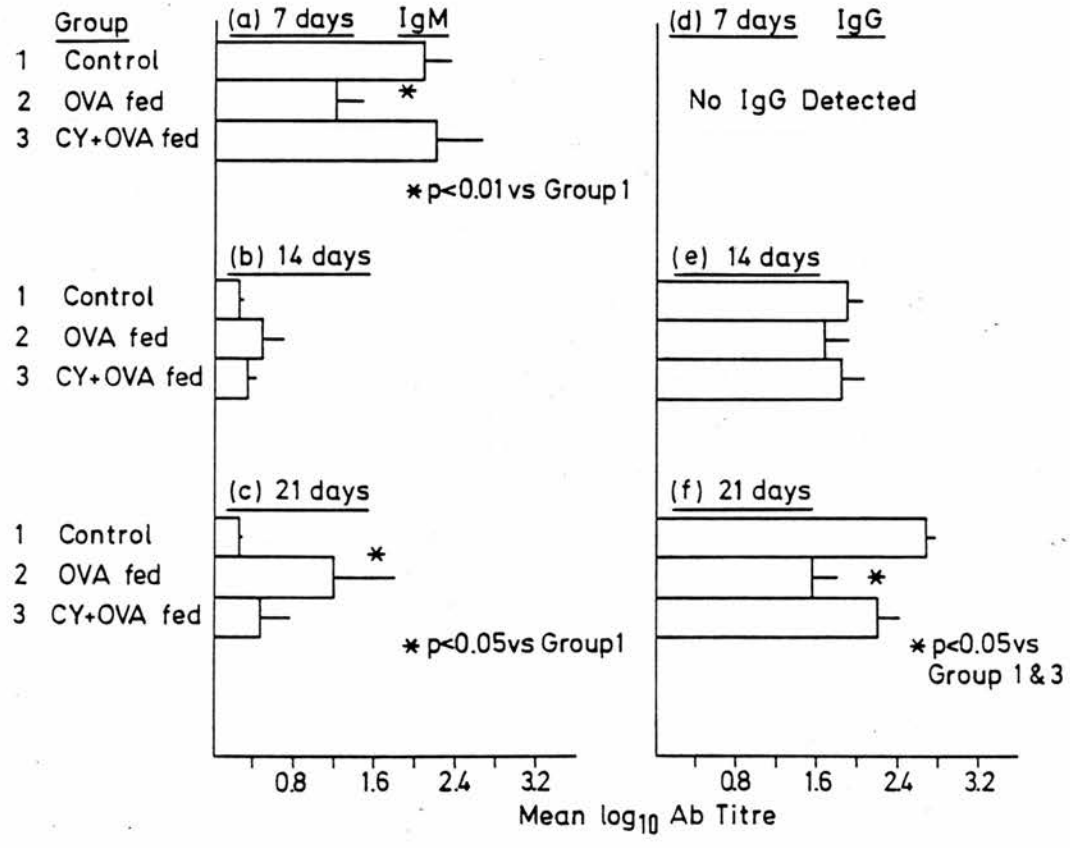

Fig. 11.3. Effect of CY on the induction of tolerance by feeding 25 mg OVA to BALB/c mice. Serum haemagglutinating IgM and IgG antibody levels 7, 14 and 21 days after 2 mg OVA in CFA i.p. Mice were fed 25 mg OVA 14 days before immunisation. Bars represent mean $\log_{10}$ antibody titres + 1 s.e.m. for each group of 6-8 mice.

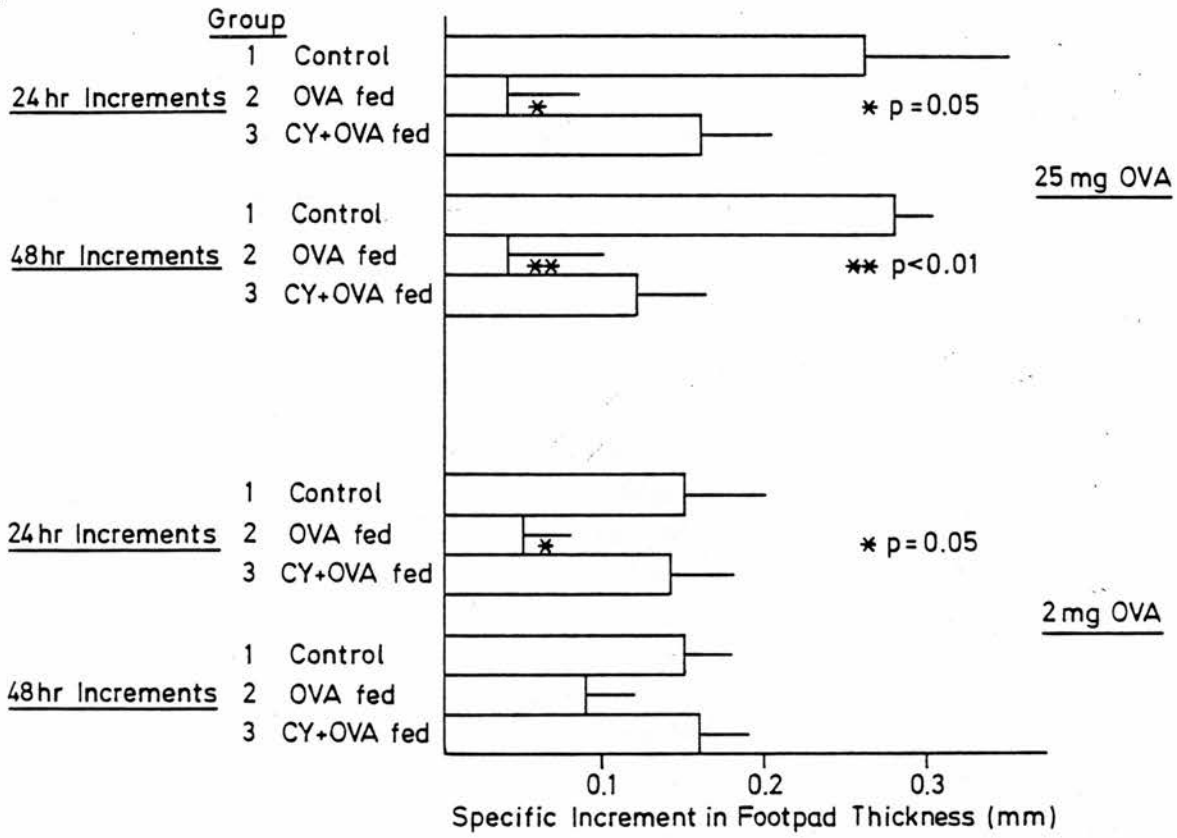

Fig. 11.4. Effect of CY on the induction of tolerance by feeding 2 or 25 mg OVA to BALB/c mice. Systemic CMI responses 3 weeks after 100 µg OVA in CFA i.d. in CY treated OVA fed, OVA fed mice and in control mice. Results are mean specific increment in footpad thickness 24 and 48 hours after 100 µg OVA in saline i.d. + 1 s.e.m. (8 mice/group). (p values in mice fed 25 mg OVA are vs. controls).

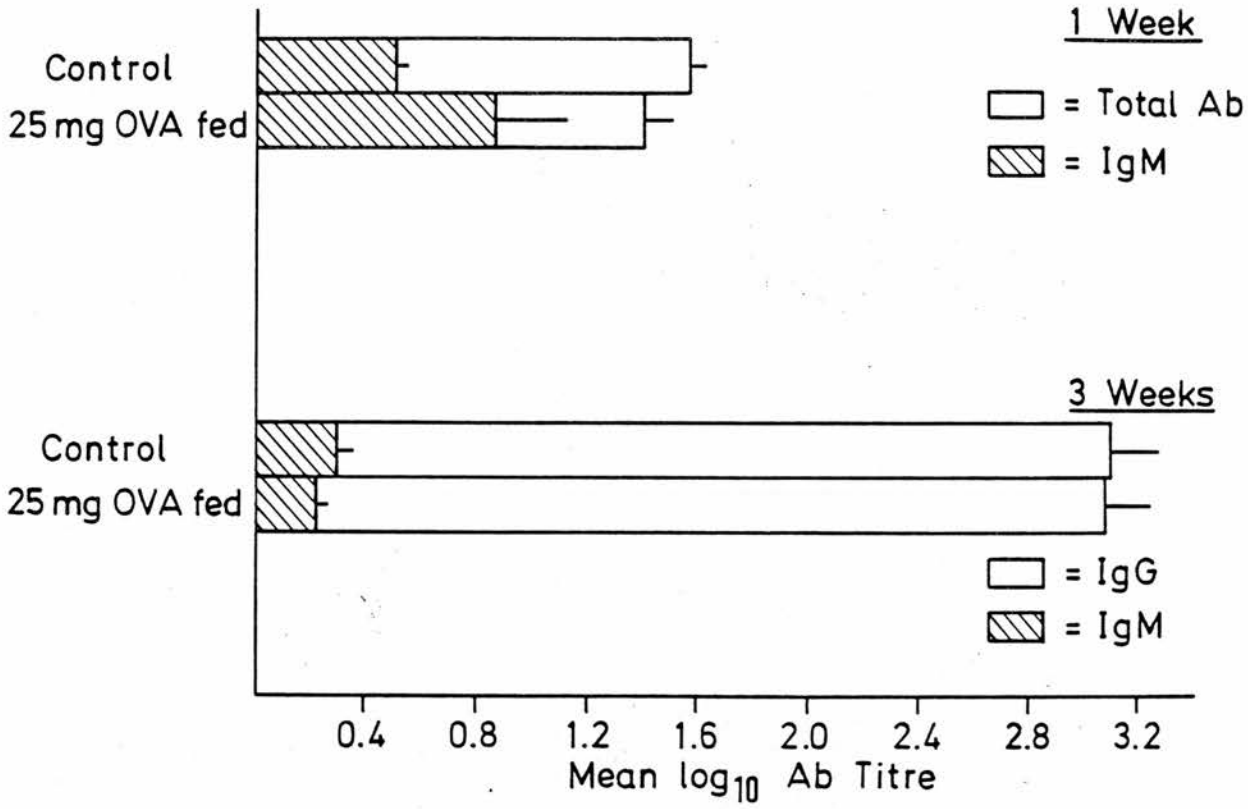

Fig. 11.5. Antigen-specificity of induction of tolerance by feeding OVA to BALB/c mice. Serum haemagglutinating anti-HSA responses in mice fed 25 mg OVA 14 days before immunisation with 2 mg HSA in CFA i.p. and in controls. Bars represent mean $\log_{10}$ antibody titres + 1 s.e.m. for each group of 6-8 mice. No significant differences were observed.

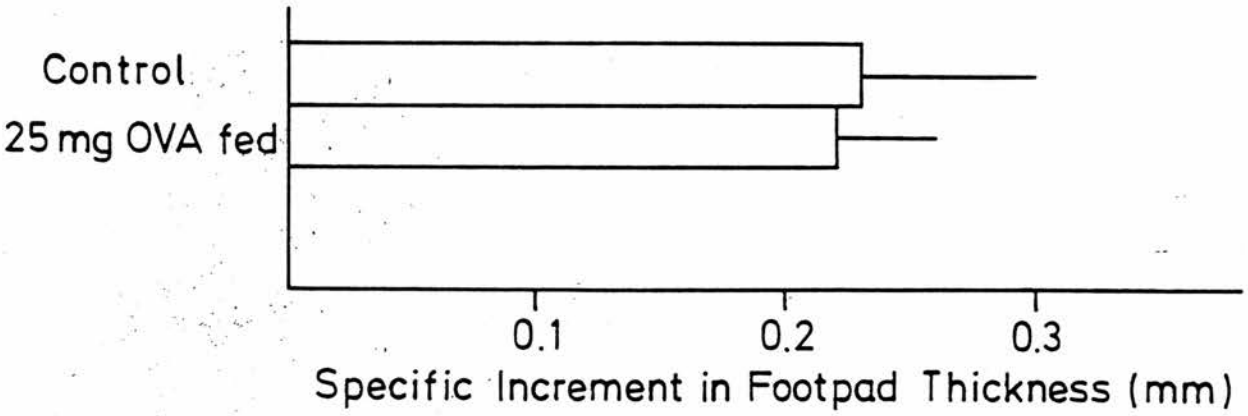

Fig. 11.6. Systemic CMI responses to HSA in mice fed 25 mg OVA and immunised with 100 µg HSA in CFA i.d. Bars represent mean specific increment in footpad thickness 24 hours after 100 µg HSA in CFA i.d. + 1 s.e.m. (8 mice/group). No significant differences.

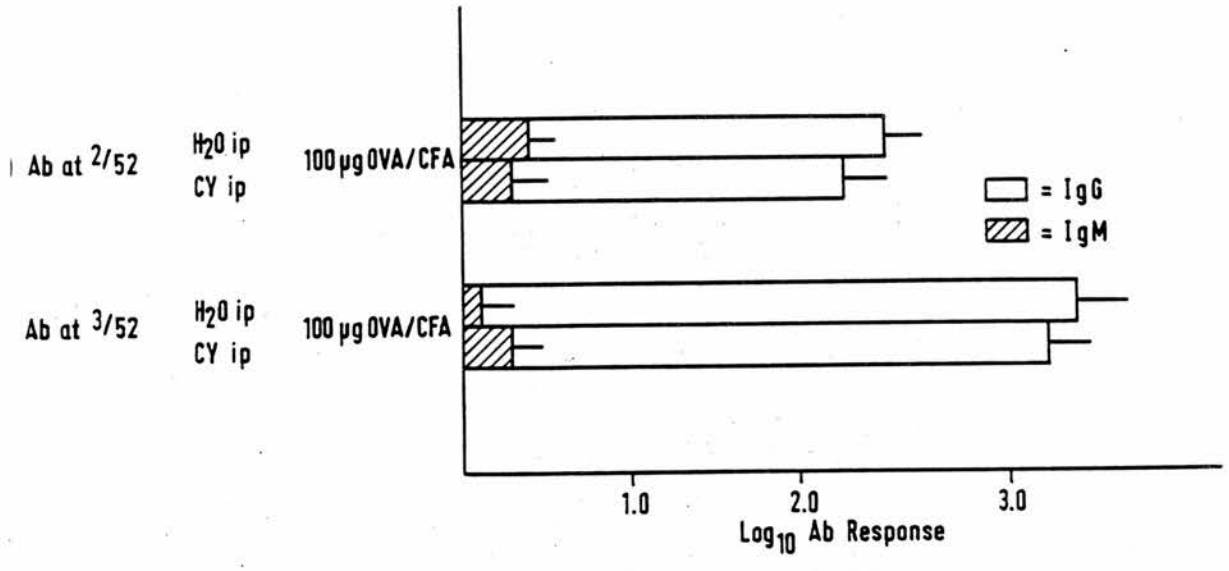

Fig. 11.7. Effect of CY on immunity to OVA. Serum haemagglutinating IgM and IgG responses in mice given CY 16 days before 100 µg OVA in CFA i.d. and in immunised controls. Bars represent mean $\log_{10}$ antibody titres + 1 s.e.m. for each group of 8 mice. No significant differences were observed.

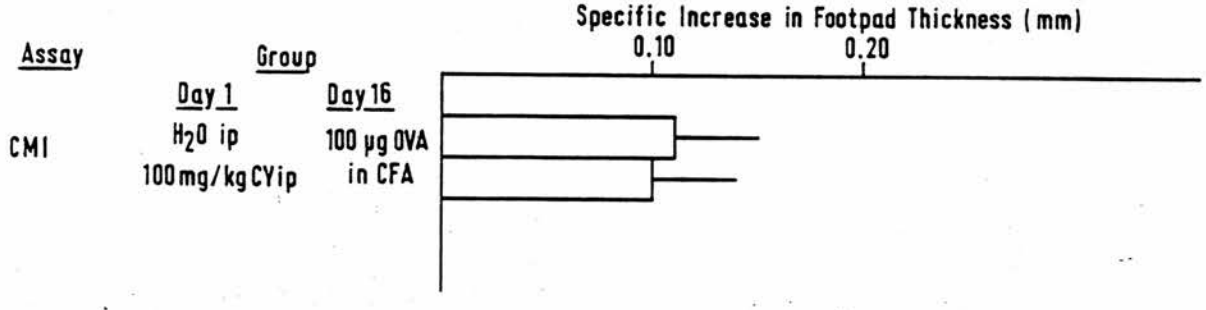

Fig. 11.8. Effect of CY on immunity to OVA. Systemic CMI responses in mice given CY 16 days before 100 µg OVA in CFA i.d. and in immunised controls, 3 weeks after immunisation. Results are mean specific increment in footpad thickness 24 hours after 100 µg OVA in saline i.d. + 1 s.e.m. No significant differences.

CHAPTER 12

EFFECTS OF CYCLOPHOSPHAMIDE ON THE ARCHITECTURE OF THE SMALL INTESTINE AND ITS LYMPHOID TISSUES

Introduction

In the experiments described earlier, cyclophosphamide was used in its role as an inhibitor of suppressor T cell function in mice. However, like other cytotoxic agents, CY may damage all rapidly dividing tissues including the small intestinal epithelium. In any study of the immunological and morphological consequences of oral immunisation in CY treated animals it is therefore essential to assess carefully the effects of CY itself on the mucosa. In this Chapter I have performed parallel studies on the morphology and cell kinetics of the jejunal mucosa and on the structure of the GALT in CY treated mice so that the results of previous chapters can be more accurately interpreted. These features were examined in some detail by microdissection and light microscopy and also by transmission and scanning electron microscopy. For this chapter I have selected only those results which I consider to be of particular relevance to my work on oral immunisation with ovalbumin. (To present the results in full would create an imbalance in the thesis).

While CY does not appear to damage mature enterocytes directly (Waldeck 1972; Ecknauer & Löhrs 1976), it does affect crypt cells and may produce secondary deficiencies of brush border enzymes (Hartwich et al 1978). Theoretically, such epithelial injury could interfere with handling of protein antigens and the effects of CY on intestinal immune responses could be due to this process. In addition, CY has profound effects on epithelial cell kinetics and I had used studies of cell kinetics to measure mucosal CMI. It was essential,

therefore, to follow alterations in mucosal architecture and CCPR after treatment with CY in the same dose as I had used in earlier experiments. Furthermore, I have examined changes in IEL count in CY treated mice, since the IEL count was also used to assess mucosal CMI.

In the lymphoid tissues, CY depletes B cell areas with relative sparing of T cell areas in both peripheral lymphoid organs and Peyer's Patches (Turk & Poulter 1972; Mazigh et al 1979). There have been no previous studies on the fate of antigen-processing structures of the GALT in CY treated animals, although protein uptake by the bursal epithelium is depressed by CY in chickens (Sachs, Beezhold & van Alten 1979). The lymphoepithelial structures of the Peyer's Patches are thought to be responsible for antigen uptake in mammals (Owen 1977) and I have paid particular attention to these structures in the present light and electron microscopic studies of the GALT in CY treated mice.

In all the experiments reported here, it was of special interest to learn the consequences of CY treatment on the intestine 48 hours after administration of the drug, since this was the time of OVA feeding in earlier experiments. In addition, mucosal architecture was examined 6 weeks after CY since this represented the time when orally immunised mice were sacrificed for mucosal assessment. In the next chapter, the effects of CY on antigen uptake by the gut are described.

Experiments and Results

Female BALB/c mice were given 100 mg/kg CY i.p. and the jejunum removed for microdissection and histology 1,2,4,7,14, 21 and 42 days thereafter. To avoid an influence of colchicine

on E.M. appearances and on lymphoid tissue architecture, separate groups of 4 mice were killed 1,2,4,6 and 12 days after CY for examination of lymphoid organs and all E.M. studies.

General Appearances

During the course of these studies, few systemic effects of CY were observed. Animals showed no evidence of weight loss, haematuria or frank diarrhoea. However, faecal pellets were softer than normal and the mice easier to handle in the first 48 hours after treatment.

Small intestinal morphology

Macroscopic features: 24 hours after CY, the jejunum was thinned and there was an apparent increase in mucus. These changes were less noticeable by 48 hours and resolved quickly thereafter.

Histology: Few significant abnormalities could be demonstrated on histological examination of the jejunum. Of these, the most prominent was the presence of dense, basophilic inclusion bodies in the crypts which were most numerous 24 hours after CY (Figs. 12.1 & 2). By 48 hours after CY, these were less in number and were virtually absent from this time. In all cases, the crypt cells themselves appeared normal. Villus epithelial cells showed slight subnuclear vacuolation until 4-6 days after CY, but were otherwise normal. The overall structure of the villi was never altered. Depletion of lymphoid cells in the lamina propria was marked in most mice examined up to 4 days after CY. Thereafter, repletion of the lamina propria became apparent.

Morphometric studies of the jejunum

Control animals were studied at the start of the experiment and a second group at 28 days. Crypt length (Fig. 12.2), and CCPR (Fig. 12.4) remained constant during the study while villus length decreased over the period (Fig. 12.3).

Experimental Animals

Villus length: There was a steady fall in villus length in the first 7 days after CY, reaching a minimum of 456.6 $\pm$ 35.4m on day 7 (Fig. 12.3). Thereafter, villus length recovered and was comparable to controls from day 14 onwards.

Crypt length: On day 1 there was a small, but significant decrease in crypt length to 99.6 $\pm$ 2.2 mμ compared to controls 104.9 $\pm$ 3.3 mμ ($p < 0.01$). This recovered by 2-4 days and significant crypt lengthening was observed on day 7 (118.2 $\pm$ 6.9 mμ). This was not detectable 14 days after CY and normal values were then maintained until the end of the study. (Fig. 12.2).

Crypt cell production rate: The most dramatic effects of CY on the small intestine were on the kinetics of crypt cell proliferation (Fig. 12.4). After a profound fall in CCPR at 24 hours (1.2 vs 4.8 in controls) a rapid, rebound increase was observed by 48 hours. At this time, CY treated mice had a CCPR of 13.2 ($p < 0.02$). This was the maximum CCPR attained during the experiment, but the CCPR remained significantly elevated until day 14 (8.7 $p < 0.025$). Thereafter, the CCPR fell to 2.5 on day 21 and was normal at 42 days.

These results indicate that the principal alterations in mucosal morphology are secondary to the effects of CY on crypt cell proliferation kinetics.

Transmission electron microscopy of the small intestine

Ultrastructural examination of the jejunum from control animals revealed the characteristic maturation of enterocytes as they migrated from the crypts onto the villus. Intra-epithelial lymphocytes were observed at all levels of the villus and the lamina propria contained large numbers of lymphocytes, plasma cells and eosinophils. Macrophages, basophils and mast cells were observed less often.

Cyclophosphamide treated mice: Once again, the crypts were the principal site of CY-induced alterations. The inclusion bodies seen on light microscopy were prominent in all tissues examined 24 hours after CY, but were less numerous thereafter and absent after 48 hours. The inclusions consisted of cellular material of unidentifiable origin and were frequently found lying within lysosomal structures inside normal crypt cells (Fig. 12.2). The crypt cells themselves appeared normal throughout the study other than exhibiting features consistent with the alterations in cell kinetics.

The majority of villus enterocytes were also undamaged by CY. However, from day 2-12, cells at the base of the villus and crypt mouth had evidence of mitochondrial damage (Fig. 12.5). This consisted of mitochondrial dilatation $\pm$ disruption and occasionally inclusion bodies were seen in these mitochondria. Mitochondrial damage was variable in extent and degree and the majority of cells had normal mitochondria at all times. These changes became less severe at later times in the study. Similar changes could occasionally be seen in control mice, but were not as prominent and did not show the same site

distribution. Otherwise villus enterocytes were normal, with no visible damage to junctional complexes or the microvillous border.

The pattern of depletion and repopulation of the lamina propria seen on light microscopy was confirmed by E.M. examination. In addition, the number of IEL was decreased 24 hours after CY but recovered rapidly thereafter.

Effects of cyclophosphamide on the morphology of the GALT

Histology: The alterations observed in the MLN and Peyer's Patches of CY treated mice were similar to those seen in the spleen and were consistent with previous studies. Progressive depletion of B cell areas became maximal 4 days after CY with subsequent regeneration and fibrosis occurring from day 6 onwards. T-dependent areas in all tissues were relatively spared by the drug and regeneration began in these areas at an earlier time. This early recovery was parallelled by massive regeneration of the thymic cortex from day 4 onwards.

In the Peyer's Patches, CY-induced damage was similar but appeared less than in other tissues and recovery was quicker (Fig. 12.6). As far as could be assessed, the lymphoid cell populations of the dome and dome epithelium were unaffected by CY.

Intraepithelial lymphocytes after cyclophosphamide

IEL counts in the jejunum were performed in parallel with histological studies of the jejunum. Fig. 12.7 shows that there was an immediate fall in IEL count 24 hours after CY to 7.6 $\pm$ 1.2 IEL/100 Epithelial cells (Controls 14.1 $\pm$ 1.9).

This had returned to control levels by 48 hours and was normal thereafter. The IEL in CY treated mice were of normal histologic and ultrastructural appearance throughout.

Ultrastructure of Peyer's Patches after cyclophosphamide

In control mice, the dome epithelium contained many lymphoid cells and enterocytes and some macrophage-like cells. M cells were characterised by sparse surface folds in clear contrast to the regular microvillus border of columnar cells and were found rarely in control specimens. The deeper, lymphoid areas of the Patches showed the characteristic appearance of organised lymphoid tissues.

Cyclophosphamide treated mice: The enterocytes in the dome epithelium showed changes after CY which were similar to those of villus epithelial cells. Mitochondrial damage was however, less severe and found less consistently. Epithelial M cells rarely showed this kind of damage at any time, even when adjacent epithelial cells were damaged. (Fig. 12.8). In addition, surface protrusions of the M cells were more prominent and more frequently seen from day 2 onwards. In contrast to control animals it was easy to identify several M cell protrusions in each CY treated mouse. In the sections examined it was not possible to trace the entire course of these cells throughout the depth of the epithelial layer. It was not clear whether there was a real increase in M cell numbers. Epithelial lymphoid cells appeared unaltered in number and structure after CY.

Scanning Electron Microscopy of the jejunum and Peyer's Patches in cyclophosphamide treated mice

The normal appearance of a Peyer's Patch and its surrounding jejunal villi is shown in Fig. 12.9, while Fig. 12.10 shows an area containing several columnar cells and one M cell. SEM examination confirmed the relative lack of M cells in control mice.

After CY, the overall surface area of the dome epithelium appeared similar to controls at all times. Examination of the surface at higher magnifications revealed a large number of M cells in all CY treated mice, confirming the impression obtained by T.E.M. (Fig. 12.11). Although difficult to quantify accurately, M cells appeared to comprise a greater proportion of the total epithelial cell population as well as an increased number/area. In addition, the surface folds were more prominent and hyperplastic than in controls. These features were apparent 24 hours after CY and maximal on days 2-6.

Major alterations in villous structure were not found in these studies. However, 24-48 hours after CY, some villi showed evidence of lateral collapse. This may have been due to the hypocellularity of the lamina propria seen on sectioned material.

Summary and Conclusions

The results of these experiments indicate that cyclophosphamide has minor, but significant effects on the small intestinal mucosa and its lymphoid tissues. These may be

prolonged for up to 14 days after a single dose of 100 mg/kg.

In the jejunal mucosa, CY affected principally the rapidly dividing crypt cells. Its action on cell proliferation was evidenced by the initial inhibition of CCPR which was followed by a rebound recovery in crypt cell mitotic activity which persisted for at least 14 days. Electron microscopy and histological examination revealed evidence of crypt damage in the first 24-48 hours after CY, but recovery occurred rapidly after this time. Damage to villous epithelial cells was restricted to those cells in the basal regions of the villus, and was confined to alterations in mitochondrial appearance. These were present at all times after 24 hours and considerable variation in extent and severity was observed. The significance of these lesions is not clear.

CY was shown to exert its principal effects on the B cell areas of lymphoid tissues, with maximal damage by day 4 and substantial recovery 6-12 days after CY. The GALT showed a similar pattern of events and the outstanding finding in the Peyer's Patches of CY treated mice related to the dome epithelium. Here, both T.E.M. and S.E.M. indicated that the specialised M cells of the dome epithelium were relatively spared by the drug. Indeed, it appeared that CY had induced an increase in number and activity of these cells, while the columnar enterocytes showed minor changes similar to those in villous cells.

In contrast to the effect of CY on the organised lymphoid tissues of the GALT, the IEL showed a rapid return to normal

levels after CY. In addition, destruction of IEL could not be demonstrated at any time. The rapid recovery of the IEL was more similar to the pattern seen in the thymus, and the results are consistent with the IEL being derived from a pool of rapidly dividing T cells.

These results are important to my work from two points of view. Firstly, they indicate that CY alone had no residual effects on the parameters of mucosal CMI when measured at the time when OVA immunised mice were sacrificed for intestinal examination (40 days after CY). However, they also show that consistent alterations were present in the mucosa 48 hours after CY, when mice were normally fed OVA for the first time. It is possible that this mucosal injury may have contributed to the observed effects of CY on oral immunisation with OVA.

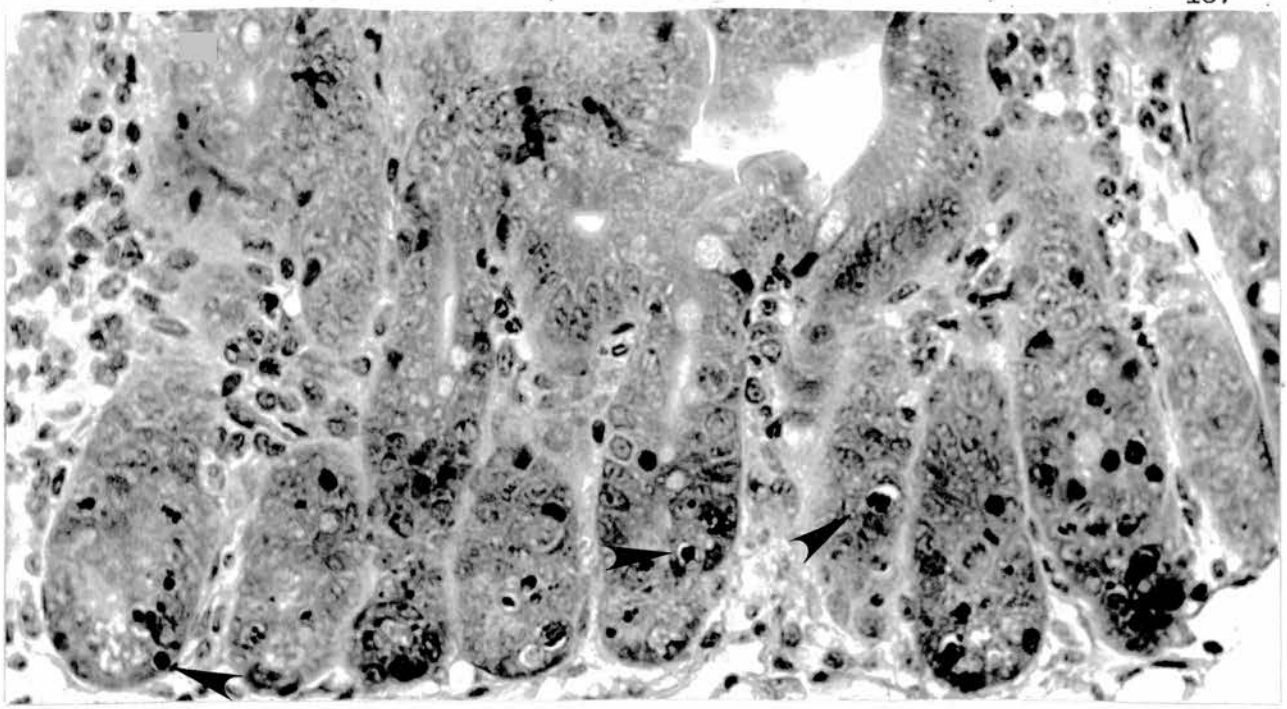

Fig. 12.1. Light microscopic appearances of mouse jejunum 24 hours after CY. Large numbers of inclusion bodies are seen in the crypts (arrows)(H & E x 320).

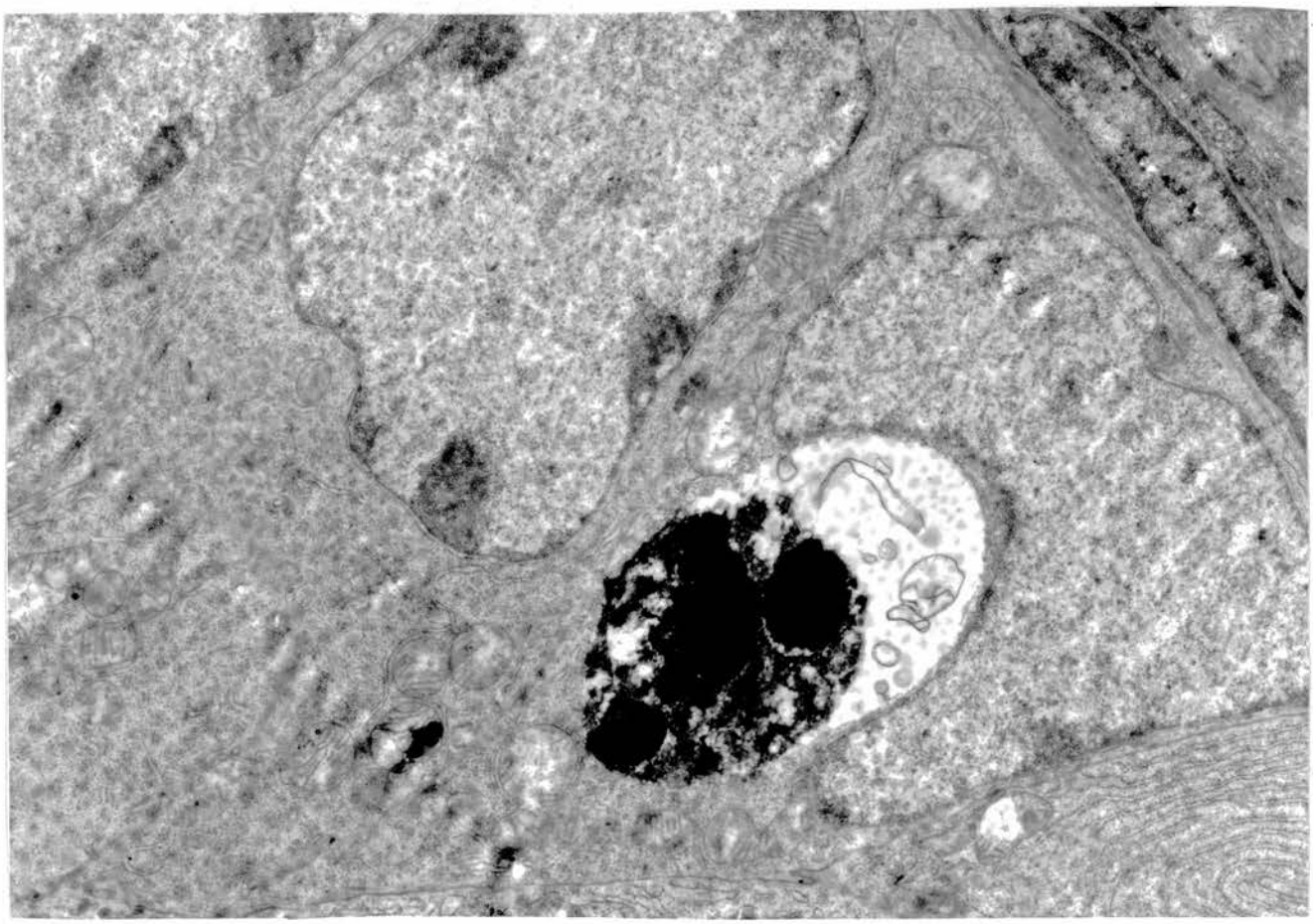

Fig. 12.2. Electron micrograph of crypt cell inclusion body which has been engulfed by a crypt cell in mouse jejunum, 24 hours after CY (x 6300).

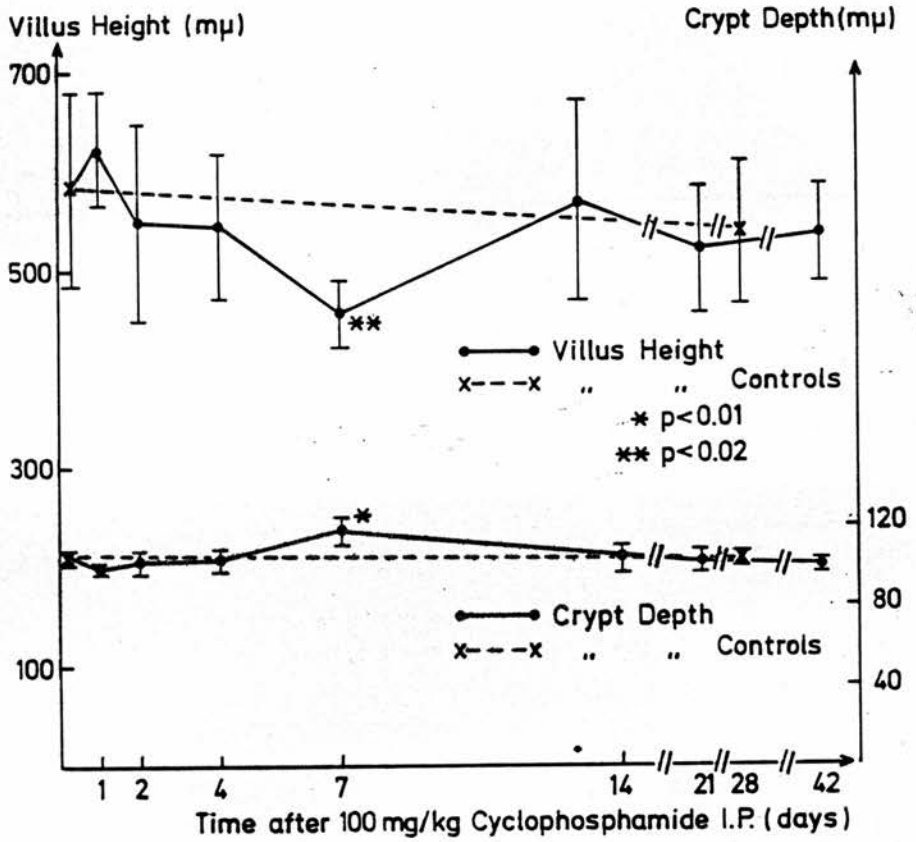

Fig. 12.3. Effect of CY on mucosal architecture of BALB/c mice. Villus and crypt lengths at intervals after 100 mg/kg CY and in age-matched controls. Bars represent means ± 1 s.d. for each group of 6 mice.

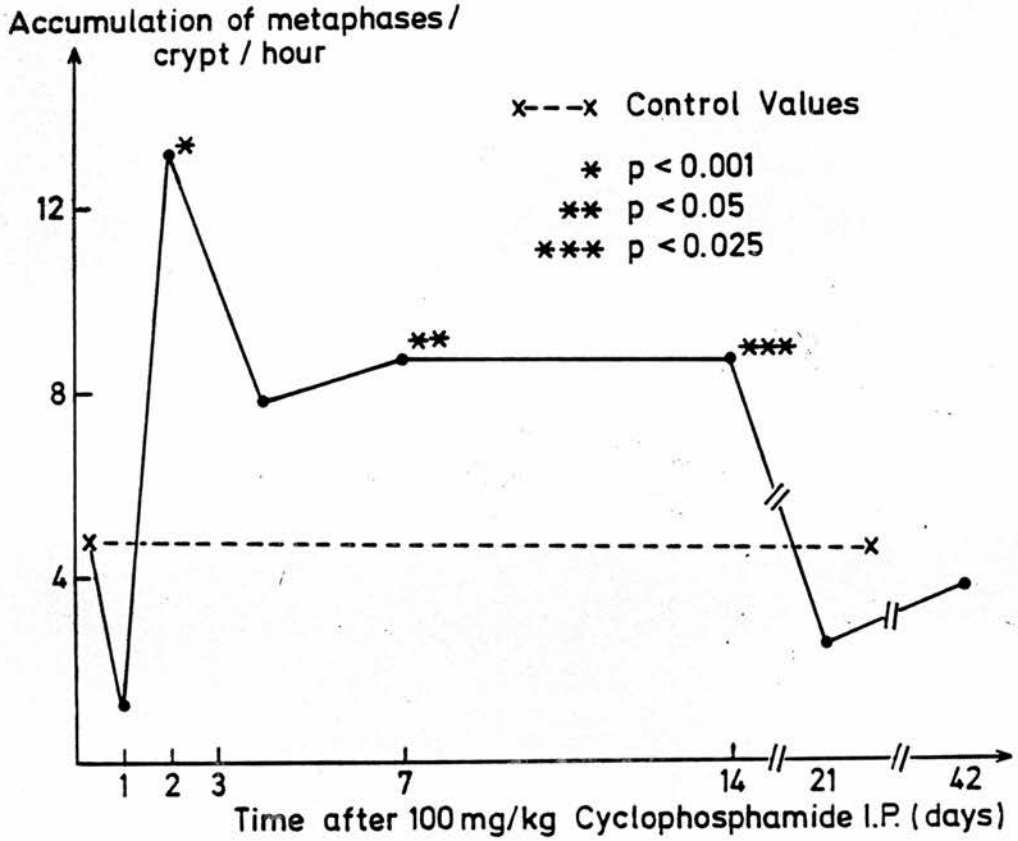

Fig. 12.4. Effect of CY on mucosal architecture of BALB/c mice. Crypt cell production rate at intervals after CY and in age-matched controls. 6 mice/ group.

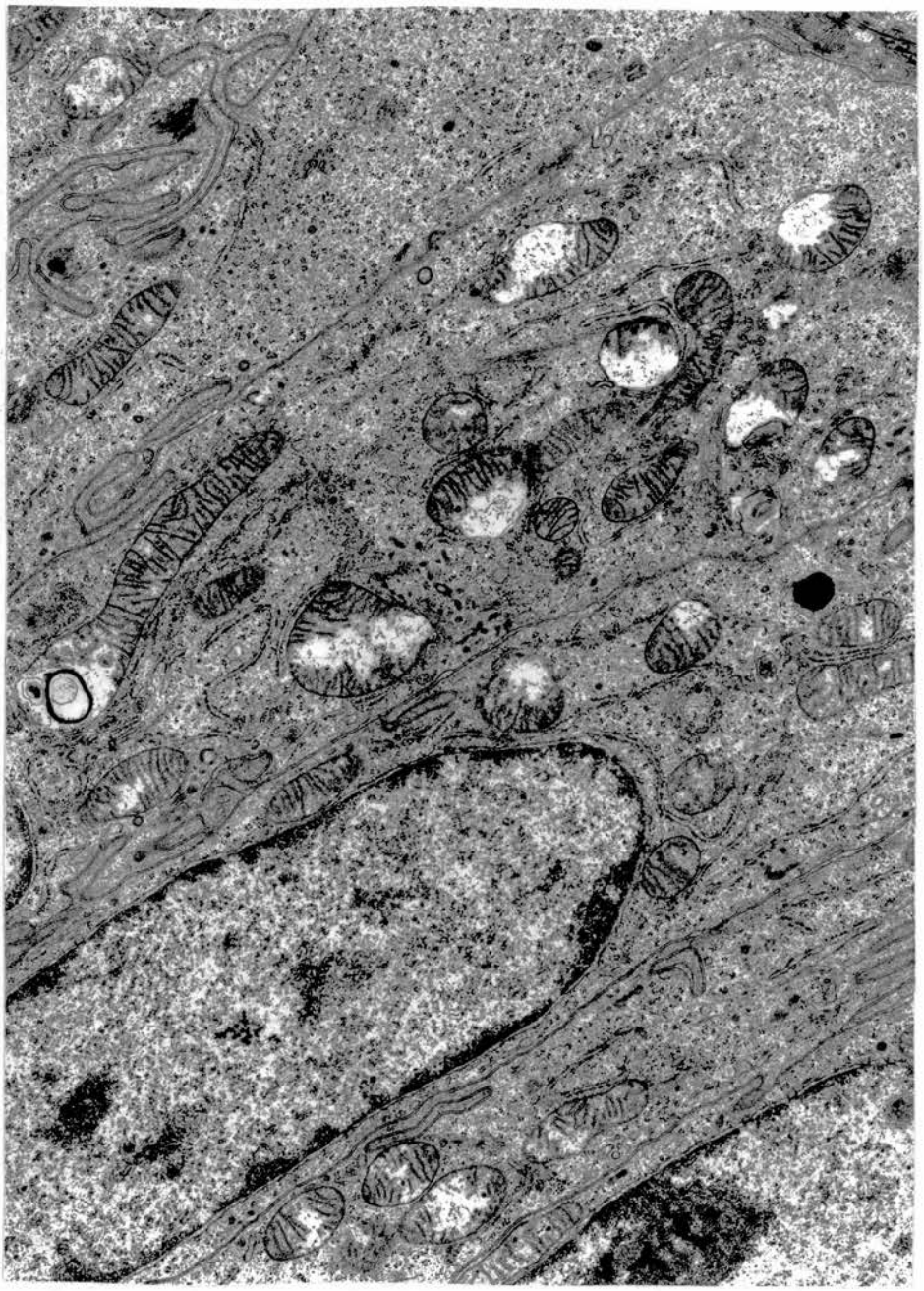

Fig. 12.5. Mitochondrial damage in jejunal crypt cells 48 hours after CY. Note that in damaged cells, some mitochondria still appear normal (x 7800).

Fig. 12.6. Light microscopic appearance of Peyer's Patch 4 days after CY. There is depletion of B cell areas (B) with relative sparing of T-dependent areas (TDA). (C = crypts). (H & E x 160.)

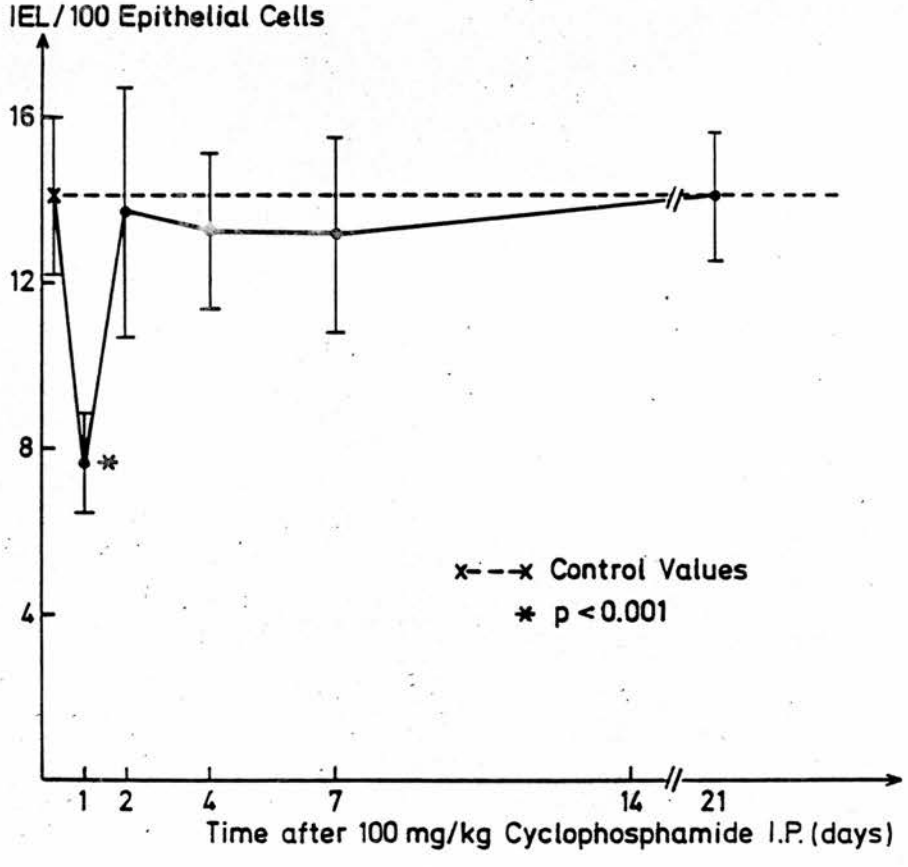

Fig. 12.7. Effect of CY on intraepithelial lymphocyte counts in jejunum of BALB/c mice. Bars represent means ± 1 s.d. for each group of 6 mice.

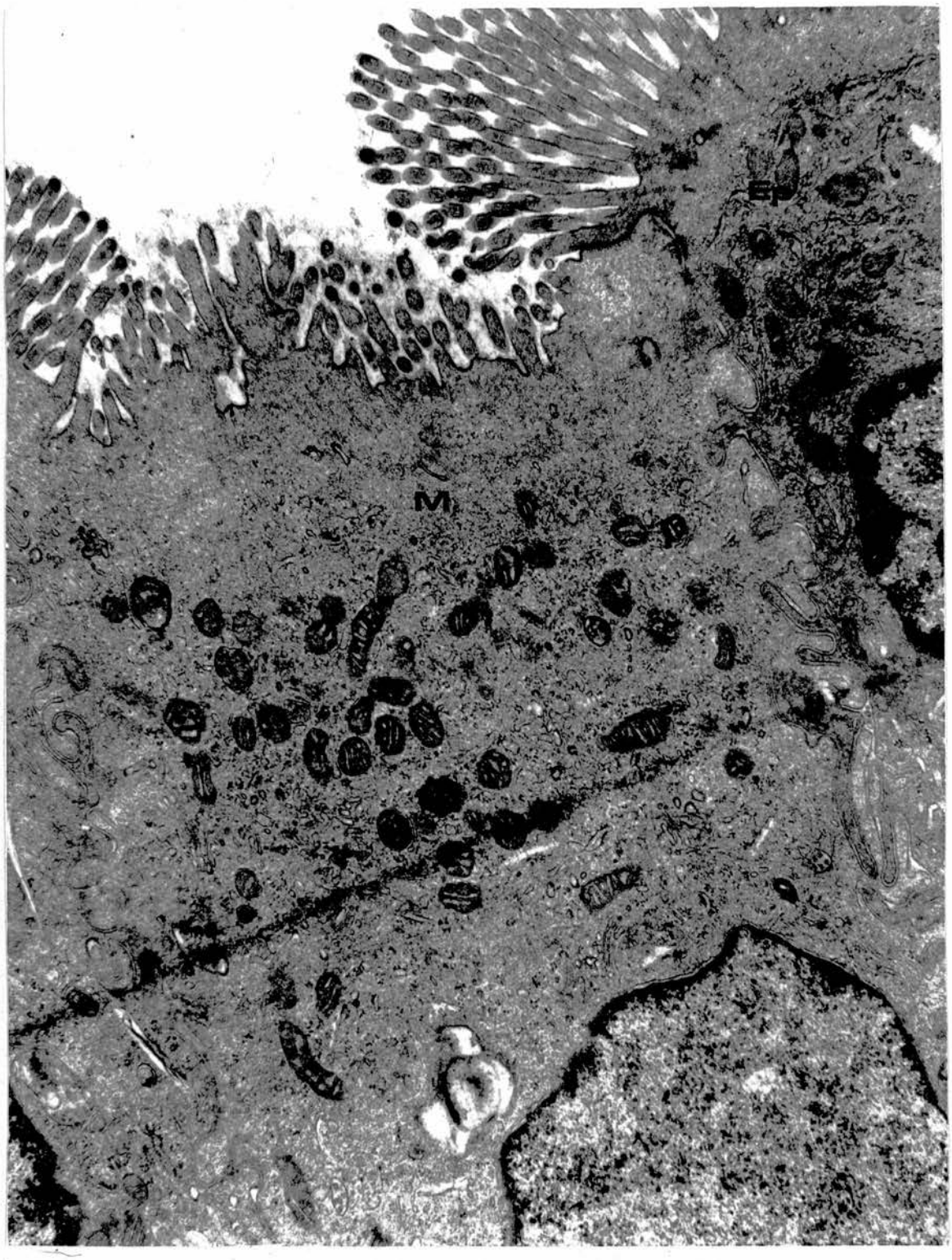

Fig. 12.8. Transmission electron micrograph of M cell in Peyer's Patch 2 days after CY. M cells are easily identifiable by their short surface folds in comparison to the long microvilli of surrounding epithelial cells (Ep). Mitochondrial damage was rarely observed in M cells (x 11,000).

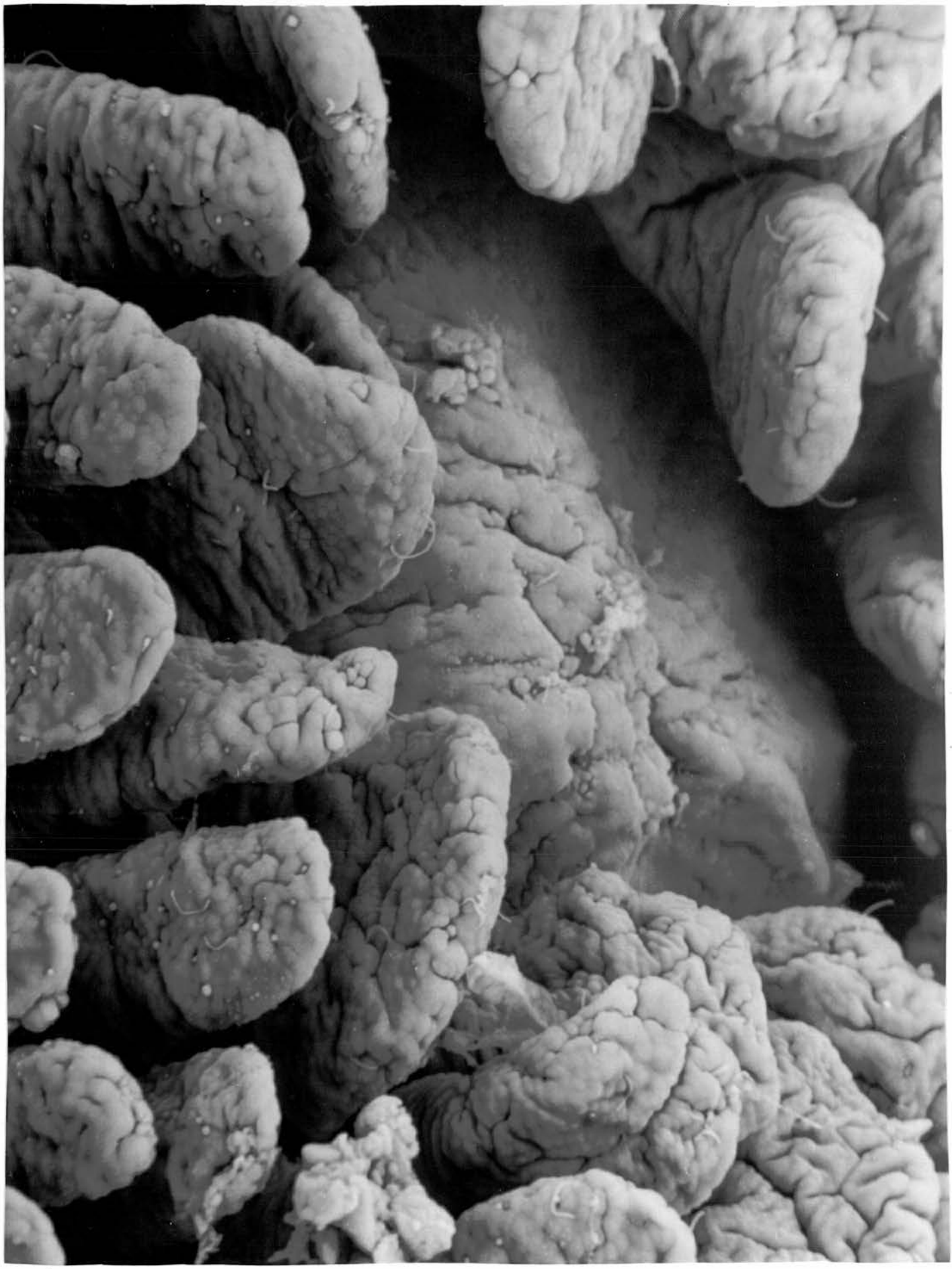

Fig. 12.9. Scanning E.M. appearance of the mucosal surface of a normal Peyer's Patch with surrounding villi. (x 197).

Fig. 12.10. Scanning electron micrograph of the dome epithelium of a normal Peyer's Patch. An M cell (arrow) is clearly identifiable from the surrounding, hexagonal enterocytes (x 1500).

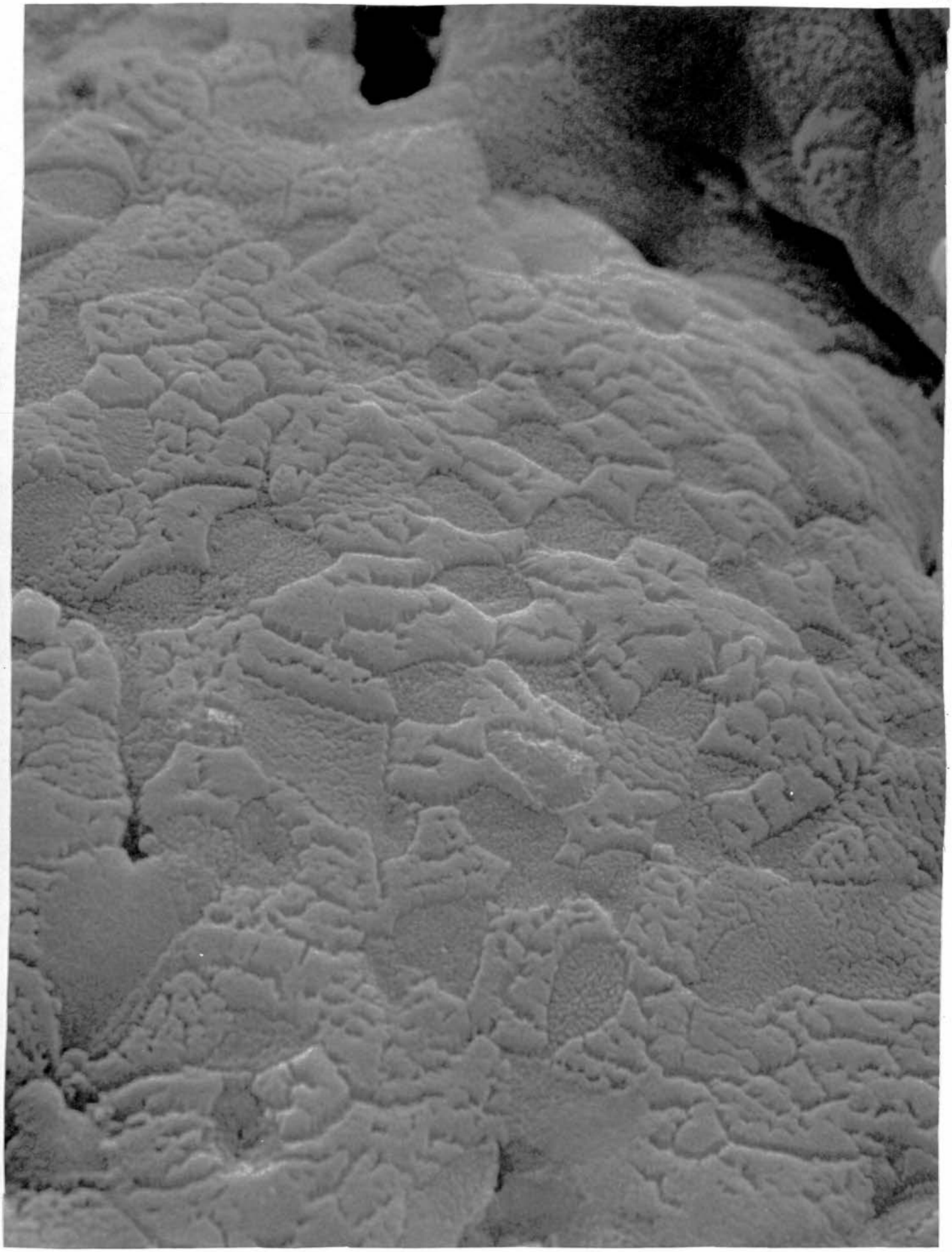

Fig. 12.11. SEM appearance of the dome epithelium of a Peyer's Patch 6 days after CY. Compared to the Peyer's Patch in control mice (Fig. 12.10), M cells were observed very frequently after CY treatment. (x 1500).

CHAPTER 13

UPTAKE AND PROCESSING OF OVALBUMIN BY THE INTESTINE OF CYCLOPHOSPHAMIDE TREATED MICE

Introduction

As discussed above, the effects of CY pretreatment on the immune responses to fed ovalbumin could have been due to altered uptake and processing of proteins by the gut mucosa. In my experiments, mice were always fed OVA two days after CY and, in Chapter 12, minor but consistent epithelial abnormalities were found in the jejunum at this time. It was essential therefore to investigate the effect of CY on the intestinal processing of OVA.

There are several mechanisms by which CY could alter immune responses to fed antigen via an effect on the mucosa. Firstly, the CY-damaged intestine might allow increased uptake of antigenic material, allowing local or systemic priming rather than tolerance. Alternatively, defective uptake of protein could result in diminished absorption of tolerogens. Finally, qualitative changes in antigen-handling could alter the nature of the antigenic molecules absorbed. This might disrupt the balance between the amounts of immunogenic and tolerogenic protein present after feeding. The aim of the experiments described in this chapter was to investigate these possibilities.

This objective was achieved in two ways. The total amount of antigenic OVA was measured in the serum of CY treated mice after oral administration of OVA in the first experiment. In a further experiment, the immunogenic properties of the absorbed material were assessed by adoptive transfer into syngeneic recipients.

Experiments and Results

Serum levels of ovalbumin after feeding

Serum was obtained from mice 1 hour after feeding 100 mg OVA and one group of mice received 100 mg/kg CY two days before feeding. The sensitivity of the haemagglutination inhibition assay used to test sera for the presence of OVA was shown earlier to be 1 µg/ml OVA and the concentrations of OVA in sera of fed mice are shown in Figure 13.1. It can be seen that there was a wide scatter of OVA concentrations after feeding (4-1280 µg/ml) in each group. The upper limit of ≑ 1 mg/ml OVA in the serum represents absorption of approximately 1% of the fed dose and this result is similar to those of other studies (Warshaw, Walker & Isselbacher 1974). The results show that CY had no significant overall effect on the uptake of OVA by the gut.

Immunological effects of serum from ovalbumin fed mice

The protocol of this experiment is shown in Fig. 13.2. Each group of donors comprised 20 BALB/c mice, each mouse receiving either 25 mg OVA (Groups 2 and 4) or 0.2 ml H_2O (Groups 1 and 3) orally. The mice were bled out from the axillary vein 50-70 minutes after feeding. In addition, Groups 3 and 4 received 100 mg/kg CY two days before feeding. The sera from each group were pooled and 0.8 ml transferred i.p. into each of six BALB/c recipients. The recipients were immunised 1 week later with 100 µg OVA in CFA intradermally, bled weekly for haemagglutinating antibodies and tested for systemic DTH by footpad testing 3 weeks after immunisation. Recipients were also assayed for antibody

levels immediately before immunisation.

Figure 13.3 shows that all recipient groups had identical levels of IgM and IgG antibody, measured 2 and 3 weeks after immunisation respectively. Thus, serum from OVA fed donors (Group 2) had no effect on the humoral immune response of naive recipients. In addition, CY treatment of the donors (Group 4) did not alter the properties of the transferred serum.

There was no evidence of priming in the recipients of OVA fed serum and this was confirmed by the absence of detectable antibody before immunisation.

A rather different pattern emerged when the CMI responses of recipients were considered (Fig. 13.4). Here, serum from OVA fed donors markedly reduced the recipients' CMI responses, measured 3 weeks after immunisation. This corresponded to 71% suppression compared to controls (Group 2 vs Groups 1 and 3, $p < 0.02$). Recipients of serum from CY treated, OVA fed mice (Group 4) also had significantly reduced CMI responses (93% suppression, $p < 0.01$) which were not significantly different to the results in recipients of Group 2.

Finally, it should be noted that CY did not alter the properties of serum from H_2O fed donors, either for humoral or cell-mediated immunity (Groups 1 and 3). There were therefore no residual effects of CY in the serum of mice given the drug two days before.

Conclusions

It is apparent from these results that CY had no effect on the quality or quantity of protein antigen absorbed by the gut. Concentrations of OVA in the serum of CY treated mice were similar to those of untreated, OVA fed mice. Furthermore, serum from OVA fed donors had identical effects on the immune responses of recipients, irrespective of whether the donors had received CY or not. Thus, the epithelial damage observed in CY treated mice has no apparent effect on the handling of protein antigens by the gut. It is possible that these results reflect the relative resistance of Peyer's Patch M cells to CY which was noted earlier.

The results of this study also confirm that humoral and cell-mediated immunity are affected differently by intestinally-absorbed antigen. Thus, serum from OVA fed donors markedly reduced the CMI response, but not the antibody response, of recipient mice. These findings support the results of Chapter 11 where it was shown that these limbs of the systemic immune response responded differently to the induction of oral tolerance and CY pretreatment, after feeding OVA.

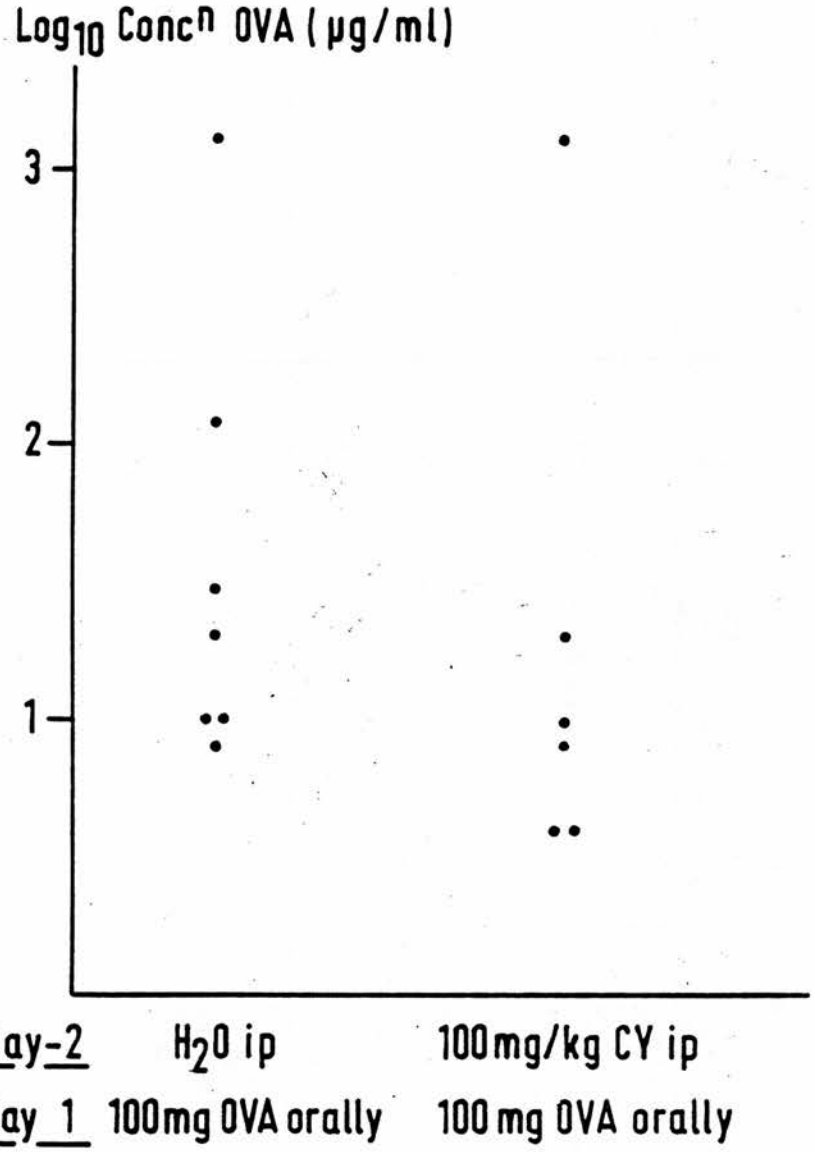

Fig. 13.1. Effect of CY on the uptake of OVA by the intestine. Serum concentrations of OVA, measured by haemagglutination inhibition, 1 hour after feeding 100 mg OVA to BALB/c mice. No significant differences between the groups were found.

Donors

Group	Day-2	Day 1	
1. (H_2O/H_2O)	H_2O i.p.	H_2O orally	Serum obtained 1 hour after feeding
2. (H_2O/OVA)	H_2O i.p.	25 mg OVA orally	
3. (CY/H_2O)	100 mg/kg CY ip	H_2O orally	0.8 ml transferred i.p. to recipient
4. (CY/OVA)	100 mg/kg CY ip	25 mg OVA orally	

Recipients

Group	Day 1	Day 7	
1	0.8 ml serum from donor Group 1	Bled for Ab + 100 µg OVA in CFA intradermally	Bled for Ab on Days 14, 21 and 28
2	0.8 ml serum from donor Group 2		
3	0.8 ml serum from donor Group 3		Footpad tested for DTH on Day 28
4	0.8 ml serum from donor Group 4		

Fig. 13.2. Protocol of experiment designed to investigate effect of CY on uptake of immunogenic OVA by the small intestine.

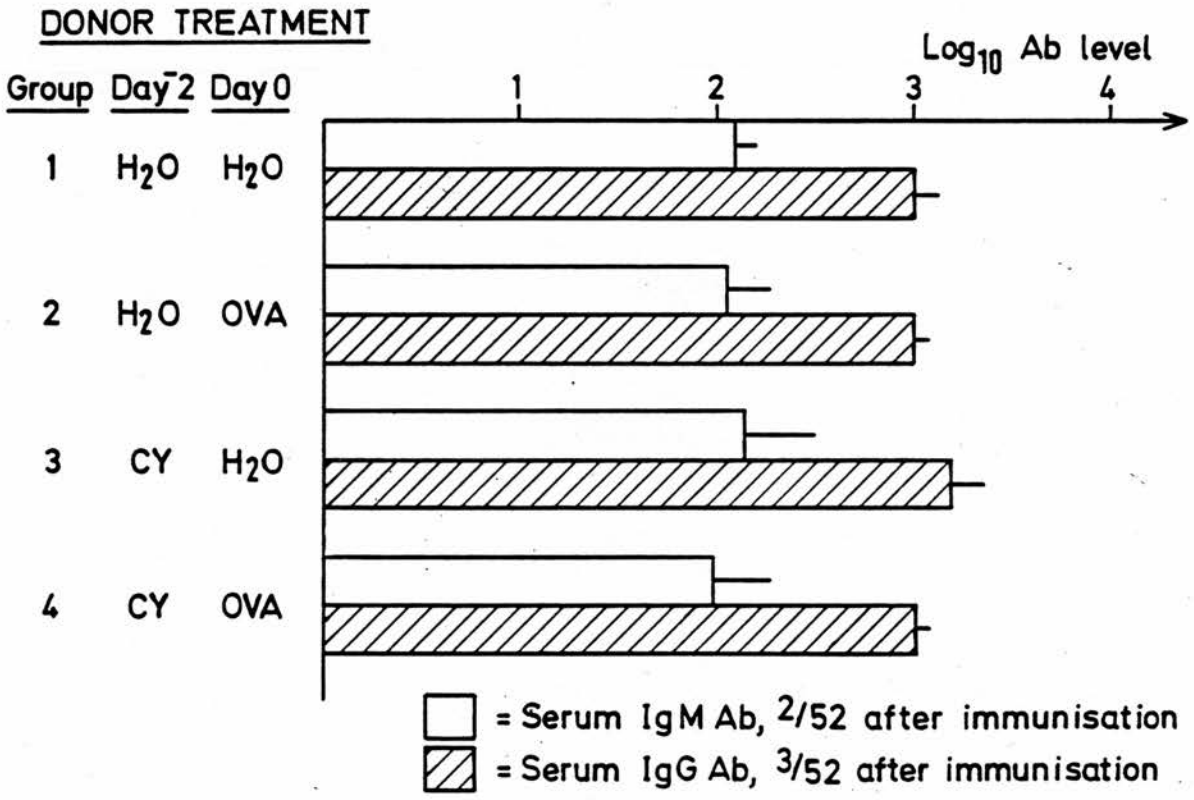

Fig. 13.3. Effect of serum from OVA fed mice and from CY pretreated OVA fed mice, on the immune response of recipients. Haemagglutinating IgM and IgG antibody levels, 2 and 3 weeks after 100 µg OVA in CFA in groups of 6 recipient mice (Mean $\log_{10}$ titres + 1 s.e.m.). No significant differences between the groups were observed. See Fig. 13.2 for details of treatment of donors and recipients.

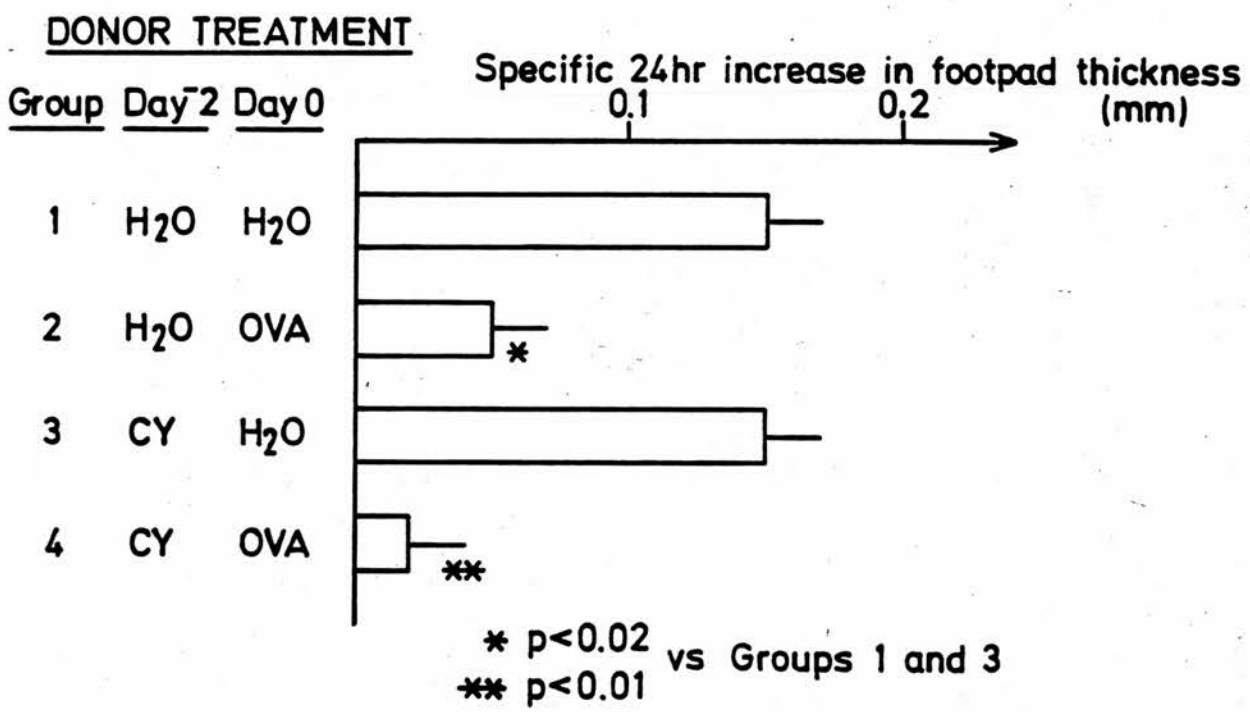

Fig. 13.4. Effect of serum from OVA fed mice and from CY pretreated OVA fed mice, on the immune response of recipients. Systemic CMI in groups of 6 recipients, measured 3 weeks after 100 µg in CFA i.d. Bars represent mean specific increments in footpad thickness 24 hours after 100 µg OVA in saline i.d. + 1 s.e.m.

CHAPTER 14

GENERAL DISCUSSION

The overall objective of this study has been achieved, in that I have been able to induce local cell mediated immunity in the gut mucosa and its lymphoid tissues to a dietary protein antigen, ovalbumin. An important aspect of these findings was that intestinal CMI could only be induced after pretreatment of the animals with cyclophosphamide before oral immunisation. The likely explanation for this is that cyclophosphamide prevented the induction of suppressor cells by fed antigen. I was also able to show that CY also inhibited the development of systemic tolerance normally resulting from feeding antigen.

While the induction of intestinal CMI to a dietary antigen required manipulation of the host immune response, mucosal CMI occurred as an integral part of a graft-versus-host reaction. In the GvHR in both adult and neonatal mice, I found significant alterations in mucosal morphology in parallel with the local immune response. The principal of these were enhancement of crypt cell mitotic activity and increased lymphocytic infiltration of the epithelium and these features proved to be reliable parameters for the assessment of mucosal CMI. Using these indices, evidence was obtained that lymphokines may be responsible for the expression of CMI in the intestinal mucosa.

Since the measurement of CMI in the mucosa and GALT was a critical component of most of the experiments described in this thesis, I shall first consider the experiments designed to assess these responses. I shall then discuss the work relating to the intestinal immune response to dietary antigens.

Mucosal alterations during the intestinal phase of the GvHR

It has proved extremely difficult to measure local CMI in the small intestinal mucosa directly, due to its contamination and inaccessibility. For this reason, measurable features of intestinal pathology have been sought as indirect indices of mucosal CMI. Previous studies had identified an increased CCPR in neonatal F_1 mice with GvHR and increases in CCPR and IEL count during allograft rejection of foetal gut (MacDonald & Ferguson 1976; 1977). However, in their work on the GvHR, these authors did not assess the alterations in mucosal architecture in parallel with direct measurement of the proliferative phase of the GvHR. I therefore proceeded to do so, and my results confirm and extend the previous, limited study of the effect of the GvHR on the mucosa of neonatal mice (MacDonald & Ferguson 1977). In addition, I have been able to examine the intestinal phase of the GvHR in the jejunum of adult mice and in antigen-free grafts of small intestine.

The earliest mucosal change in neonatal mice with GvHR was shown to be an increased IEL count within 24 hours of transfer of parental spleen cells and the IEL count continued to rise and fall in direct parallel with the Spleen Index. Increased IEL counts were also found in the jejunum of adult F_1 mice and in antigen-free grafts of gut during the GvHR. The alterations in the IEL count are therefore not merely a feature of the response by the immature gut to injury and are not influenced by bacterial contamination of the mucosa.

These findings, together with the T cell nature of the IEL (Ferguson & Parrott 1972a; Guy-Grand et al 1974; 1978; Janossy, Tidman, Selby, Thomas, Granger, Kung & Goldstein 1980) and their increase during allograft rejection of gut (MacDonald & Ferguson 1976) suggest that the increased IEL count is directly related to mucosal CMI. The present results cannot identify the cause or the source of the continued rise in IEL count in neonatal mice. Since increased numbers of IEL are also found during the GvHR in irradiated mice, it is probable that these cells are of donor rather than host origin (Guy-Grand et al 1978).

The rapid rise in IEL count in neonatal hosts is of some interest. This did not occur when F_1 animals were injected with F_1 spleen cells and this increase must be a specific consequence of the interaction between donor cells and host tissues. This idea is supported by the continuing increase in IEL count which paralleled the Spleen Index exactly. The exact role of the IEL in the GvHR is also unclear. In humans, the majority of IEL are reported to be of the suppressor T cell phenotype (Janossy et al 1980) and suppressor T cells do proliferate during the GvHR in mice (Pickel & Hoffman 1977). Nevertheless the early rise in IEL count and their close relationship with the Spleen Index would argue against this phenomenon being a proliferation of suppressor T cells.

Mucosal mast cells undergo a thymus-dependent proliferation during parasite infections of the small intestine (Ruitenberg &

Elgersma 1976; Mayrhofer 1979) and this response is transferrable using slg$^-$TDL from Nippostrongylus-immune rats (Nawa & Miller 1979). In addition, large numbers of lymphoid cells containing mast cell-like granules and bearing a T cell marker have been described in the mucosa of mice with GvHR (Guy-Grand et al 1978). It is therefore of note that prolonged hyperplasia of mucosal mast cells was observed during the GvHR in the present study. In addition, this was accompanied by an expansion of intraepithelial cells staining positively for mast cell granules. It is clear that the proliferation of mucosal mast cells played no part in the development of the other mucosal changes observed since the mast cell response was delayed in relation to the other indices. The MMC response during infection with Nippostrongylus brasiliensis is similarly delayed after the onset of worm expulsion (Miller & Jarrett 1971) and the MMC may not necessarily be effector cells in intestinal immune responses. In addition to the thymus-dependency of the MMC response to intestinal parasites, there is considerable evidence to support a close relationship between mast cells and CMI. Mast cell infiltration has been reported during a local GvHR in hamster skin (Billingham & Streilen 1968) while mast cell degranulation occurs in DTH reactions in the skin of mice (Askenase, Bursztajn, Gershon & Gershon 1980). Lymphokine-dependent mastocytosis and histamine synthesis has also been observed during the allograft reaction in mice (Dy, Lebel, Kamoun and Hamburger 1981) and recently an

Lyt $1^{+}2\bar{3}$ clone of T cells has been reported to produce a factor which stimulates in vitro differentiation of mast cells (Nabel, Galli, Dvorak, Dvorak & Cantor 1981). Thus CMI responses in the mucosa and elsewhere may specifically recruit and activate mast cells. It is tempting to speculate that histamine released by these cells may then suppress further extension of the local CMI reaction (Rocklin 1978). Alternatively, it has been suggested that MMC may themselves be derived from mucosal T cells (Guy-Grand et al 1978). The present results may either reflect recruitment of long-lived mast cells by mucosal T cells or differentiation of T cells into mast cells.

Soon after the initial rise in IEL count, neonatal mice with GvHR developed an increased crypt cell mitotic activity, confirming an earlier report (MacDonald & Ferguson 1977). This also correlated significantly with the Spleen Index and was found in both the host jejunum and in sterile, antigen-free F_1 grafts in adult mice with GvHR. These features indicate that a raised CCPR may also be a direct consequence of mucosal CMI during the GvHR. During allograft rejection of foetal gut, a similar, early increase in CCPR is found (MacDonald & Ferguson 1977), while the crypt hyperplasia which occurs during infection with Nippostrongylus brasiliensis (Ferguson & Jarrett 1975) or with Trichinella spiralis (Manson-Smith et al 1979) is thymus-dependent. Furthermore, crypt changes in the last case are preceded by migration of T lymphoblasts into the mucosa (Manson-Smith et al 1979). It is apparent

therefore that enhancement of crypt cell mitotic activity is a specific, if indirect parameter of local CMI in the intestinal mucosa.

The results of Chapters 6 and 7 indicate that the IEL count and CCPR are sensitive and reliable indicators of the mucosal CMI during the GvHR, correlating closely with the proliferative phase of the GvHR. Other studies show that these findings may be applicable to intestinal CMI in general and these parameters were therefore used in studies on the induction and expression of CMI to orally administered antigens.

Pathogenesis of mucosal lesions during the GvHR (Fig. 14.1)

Influence of intestinal bacteria

In the experiments described in Chapter 7, alterations in IEL count and mucosal architecture were found in sterile, antigen-free grafts of foetal F_1 small intestine during the GvHR and these were identical to the changes in IEL count and mucosal architecture in normally sited jejunum. This confirms other work which used less sensitive methods of assessing mucosal damage (Elson et al 1977, Van Bekkum & Knaan 1977) and indicates that direct bacterial contact is not required for the mucosal changes of the GvHR. It has been reported that an intact intestinal flora in the host may be required for damage to occur in heterotopic grafts of gut (Van Bekkum & Knaan 1977) and endotoxin may contribute to the early mortality of the GvHR (Jones, Wilson & Bealmear 1971; van Bekkum, Roodenburg, Heidt & van der Waal 1974).

During the GvHR bacterial invasion of the mucosa occurs (Woodruff et al 1976) and intestinal flora may influence both epithelial cell kinetics (Abrams, Bauer & Sprinz 1963) and host CMI (MacDonald & Carter 1979). It is possible therefore that increased absorption of bacterial products through an already damaged intestine may amplify the mucosal lesions initiated by local CMI. In this way, more severe forms of intestinal damage, including villous atrophy, may result from local CMI in the intestinal wall.

Roles of cytotoxicity and delayed-type hypersensitivity

During the GvHR in mice, large numbers of cytotoxic cells are generated by the recognition of host, H-2 coded antigens by alloreactive T cells (Cerottini et al 1971; Singh et al 1972; Fung & Sabbadini 1976). Theoretically, direct cell-mediated cytotoxicity might result in epithelial cell damage in the intestine. However, several pieces of evidence would argue against this idea. As discussed before, the initial response of the mucosa in the GvHR is enhanced crypt mitotic activity, while epithelial cell damage is only a late feature of allograft rejection of gut (Ferguson & Parrott 1973; MacDonald & Ferguson 1976). Of greater significance is my finding that foetal grafts of parental type gut are damaged during the GvHR, despite being syngeneic to the injected alloreactive spleen cells. This confirms similar reports of "bystander" damage during the GvHR in skin (Billingham & Streilen 1968), kidney (Elkins & Guttman 1969) and more recently in the small intestine (Elson et al 1977; van Bekkum & Knaan 1977). Direct cytotoxicity by donor cells cannot

account for these findings. Further evidence against a major role for cytotoxicity is the finding of identical intestinal changes in the H-2 compatible, Mls incompatible (CBA x C3H) F_1 mice with GvHR. Mls incompatibility does not generate cytotoxic cells in vitro (Peck & Bach 1974) while in the GvHR, DTH responses are induced in the absence of cytotoxicity (Wolters et al 1980). Finally, mucosal changes in the GvHR correlated closely with the proliferative phase of the GvHR, while in Chapter 10 similar alterations were found during the local CMI response to a protein antigen. There is thus strong evidence that the effector limb of mucosal CMI is related to DTH rather than the action of cytotoxic cells and it is likely that this DTH is mediated by mucosal T cells proliferating in response to foreign antigen. It would therefore be of interest to monitor the development of mucosal CMI in parallel with the presence of DTH effector cells in the host animal and its mucosa.

Pathways of mucosal T cell activation in GvHR (Fig. 14.1)

In the experiments described in Chapter 7, the epithelial elements of parental-type grafts could not have provided targets for either the induction or expression of alloreactive T cells. During growth under the kidney capsule however, foetal gut grafts become populated with host lymphoid cells (Ferguson & Parrott 1972a,b) and both F_1 and parental-type grafts will contain F_1 cells capable of stimulating the donor T cells known to populate such grafts during the GvHR (Guy-Grand et al 1978).

Proliferation of alloreactive T cells in the GvHR is stimulated by the I-A region of the H-2 complex in mice (Klein 1977) and this region is also important for the development of DTH during the GvHR (Wolters & Benner 1979). I-A bearing, recirculating host cells in the grafts may therefore provide the signal for donor T cell proliferation and development of mucosal CMI. In addition, the Peyer's Patches have recently been shown to contain I-A bearing cells (Challacombe, Krco, David & Tomasi 1979) and are important sources of gut-infiltrating cells in the GvHR (Guy-Grand et al 1978). This may provide an additional pool of activated T cells which reach the mucosa via the thoracic duct where gut-seeking T lymphoblasts are abundant in the GvHR (Sprent & Miller 1972; Sprent 1976). The present results indicate that this immigration of lymphocytes occurs rapidly after induction of the GvHR, since neonatal mice had raised IEL counts within 24 hours. The significance of this finding is reinforced by previous work which showed that specific activation and trapping of parental lymphocytes occurs within 18-24 hours after transfer into F_1 hosts (Ford, Simmons & Atkins 1975), while maximal localisation of MLN lymphoblasts in the mucosa occurs within 4 hours (Sprent & Miller 1972; Ottoway & Parrott 1980). Detailed autoradiographic studies are required to elucidate fully the migration patterns of T cells during the intestinal phase of the GvHR.

Gut epithelial cells have recently been found to bear surface I-A (Curman, Kampe, Rask & Peterson 1979) and it is

theoretically possible that these cells could provide a further site of T cell activation in the mucosa itself. Finally, random migration to the gut of T cells activated in other lymphoid organs may also contribute to the accumulation of proliferating T cells in the small intestine.

Role of soluble factors in the expression of mucosal CMI

The evidence discussed above indicates that the mucosal lesions of the GvHR are due to DTH mediated by proliferating mucosal T cells, suggesting that soluble mediators are responsible for many of the mucosal alterations observed. This is supported by the "bystander" lesions found in parental-type grafts of foetal gut, since these are likely to be secondary to the release of soluble factors by donor T cells encountering F_1 passenger leucocytes in the graft mucosa. "Enteropathic lymphokines" have already been proposed to account for the mucosal changes in intestinal CMI responses (Ferguson & MacDonald 1977) and it has been suggested that lymphokines are mainly responsible for the pathological lesions of the GvHR (Grebe & Streilen 1976). In support of this concept, lymphokines are produced by Lyt 1^+ T cells in mice (Kühner et al 1980), a subset whose activation is controlled by the I-A subregion, the part of the H-2 complex important for the proliferative response in the GvHR (Cantor & Boyse 1975; Alter & Bach 1979). It is also likely that lymphokines are essential for the mucosal alterations found when local CMI was induced to a dietary protein antigen.

These lymphokines would have several properties (Fig. 14.1). Firstly, they should have a stimulatory effect on crypt stem cell proliferation and it is possible that this effect is analagous to the influence which T cell products have on bone marrow stem cells (Schroder, Arnold & Clark-Lewis 1980). In addition, lymphokines will recruit and trap further specific and non-specific effector cells in the mucosa, including macrophages, mast cells, polymorphs and additional lymphocytes. Finally, these mediators may alter the function of the pericryptal fibroblasts thought to be responsible for the skeletal framework of the mucosa (Marsh & Trier 1974; Parker et al 1974). Lymphokines which alter the metabolism of fibroblasts in vitro have been described (Wahl, Wahl & McCarthy 1978; Postlethwaite & Kang 1979) and a mechanism of this kind would accentuate disruption of the mucosal architecture. Evidence for this type of lesion comes from the basement membrane abnormalities seen in allograft rejection of the gut (Ferguson, Carr, MacDonald & Watt 1978).

In addition to acting on cells of the immune system, lymphokines are known to act on non-lymphoid tissues including fibroblasts, osteoblasts (Horton, Oppenheim, Mergenhagen & Raisz 1974) skin (Pick, Krejci, Cech & Turk 1969), vascular tissue (Nishioka & Katayama 1978) and brain (Fontana, Grieder, Arrenbrecht & Grob 1980). As in these tissues, lymphokines released during an intestinal CMI response seem to provide the links between the lymphoid and non-lymphoid elements of

an organ which are essential factors in the repair of an organ damaged by a local immune response. As discussed in Chapter 2, there is strong evidence that lymphokines are produced in the intestinal mucosa after various schedules of oral immunisation and during allograft rejection. This is further support for a crucial role for lymphokines in intestinal immune reactions.

The question of the cells producing the postulated lymphokines is of some interest. Since mucosal CMI is associated with an increased IEL count, it might be thought that these cells are responsible for this phenomenon. In man (Janossy et al 1980) and mice (Micklem H.S., Davies, M.D.J. & Parrott, D.M.V. personal communication) the majority of IE T cells are of the suppressor/cytotoxic phenotype and it is more likely that the abundant helper/inducer T cells of the lamina propria (Janossy et al 1980) perform this function. Very recently, however, it has been found that IEL are capable of transferring DTH to a protein antigen (Shields, J.G. & Parrott, D.M.V. - unpublished observations). Thus, the nature of the mucosal effector T cell remains to be established.

Migration inhibition of lymph node cells as an assay for CMI

In the experiments described in this Chapter, it was shown that migration inhibition of lymph node cells correlated with DTH in vivo. In practice, the most reproducible results were obtained when cells were tested against 0.1 and 1 mg/ml OVA 3 weeks after immunisation and the migrating cells were predominantly lymphocytes.

The correlation of the *in vitro* assay with *in vivo* immunity was obtained by studying the development of systemic humoral and cell mediated immunity in parallel with the development of migration inhibition. When mice were immunised with 100 µg OVA in CFA, all measures of the immune response developed simultaneously. However, migration inhibition and systemic DTH occurred in the absence of serum antibodies, 1 week after 10 µg OVA in CFA, while mice immunised with OVA in IFA had excellent antibody responses but negligible DTH and no migration inhibition. Although immune complexes are reported to inhibit cell migration *in vitro* (Bloom & Bennett 1966; Carpenter et al 1968), the results indicate that migration inhibition is more closely related to systemic DTH *in vivo* rather than the antibody response. Since migration inhibition continued to develop after systemic DTH had disappeared in mice immunised with 10 µg OVA it is likely that footpad testing and migration inhibition are measuring parallel but non-identical aspects of DTH. It is possible that the results reflect the presence of locally developing immunity in regional lymph nodes rather than systemically disseminated CMI.

The results concur with previous reports of migration inhibition of lymphoid cell populations. Direct migration inhibition assays using protein antigens have been described for spleen (Rich & Lewis 1932; Carpenter et al 1968; Likhite et al 1972; Hughes et al 1980), lymph nodes (Thor & Dray 1968; Likhite et al 1972), thymus (Bakker, Engelhart, Mulder &

Hoedemaeker 1975; Hughes et al 1980) and tonsil (Mazuran, Rabatic, Sabioncello & Dekaris 1979) and have been correlated with DTH *in vivo* in several cases (Thor & Dray 1968; Likhite et al 1972; Mazuran et al 1979; Hughes et al 1980). In addition, migration inhibition of peripheral blood lymphocytes has been associated with other measures of CMI (Kowalczyk & Zembala 1978; Bradsher, Sutcliffe & Alford 1979). The present study differs from others employing lymphoid organs in some important details; firstly, the *in vitro* assay was performed in the absence of serum, since unreliable results were found in the presence of serum. Secondly, many other groups have used considerably larger concentrations of protein to induce migration inhibition, while the present test was shown to have a high level of sensitivity. Finally, I have been able to identify with some certainty that the predominant migrating cell was the lymphocyte. This confirms the report of Likhite et al and also supports recent evidence that lymphocytes are capable of migration *in vitro* (Parrott 1980). It has been reported that T lymphocytes migrate particularly well (Kowalczyk & Zembala 1978) and in my experiments, nylon wool purified T cells did show excellent migration areas. However, fluorescent analyses of migrating cells indicated the presence of B and T cells in proportions similar to those in the original lymph node. The results indicate that migration inhibition of lymphocytes is analogous to the parallel tests employing accessory cells as indicator cells and is an *in vitro* correlate of DTH.

A point of practical interest is that addition of antigen to non-immune cells was found to stimulate migration in many cases. This confirms earlier reports (Bakker et al 1975; Hughes et al 1980) but contrasts with other work in which enhanced migration of macrophages has been described as a variant form of *in vitro* CMI (Weisbart, Bluestone, Goldberg & Pearson 1976). My results indicate that this may be a non-specific effect of antigen.

This study was unable to identify conclusively the migrating cell or the cells responsible for inducing migration inhibition. In other studies, T cells accounted for the migration inhibition of human lymphocytes (Kowalczyk & Zembala 1978) and enhanced migration of mouse spleen cells (Castes, Borderie & Orbach-Arbouys 1978) and were also the predominant migrating cell in each case. Purified T cells showed excellent migration inhibition in this study, but results with "non-T cell" populations were inconsistent. While evidence was obtained to suggest that these populations were contaminated with T cells, the possibility remains that lymphoid cells in addition to T cells take part in this phenomenon. The presence of lymphokines was not demonstrated in these experiments. In addition to the factors responsible for migration inhibition of accessory cells (see Chapter 2), lymphokines which inhibit (Kowalczyk & Zembala 1978) and stimulate (Castes et al 1978) *in vitro* migration of T cells have been described. While it is likely that similar factors are operating in the assay described

here, further studies are required to elucidate fully the exact nature of the cells and mediators involved.

Influence of cyclophosphamide on small intestinal structure and function

In order to allow rational discussion of the experiments involving local intestinal immune reactions in cyclophosphamide treated mice, I shall first discuss the experiments on the effects on the small intestine and GALT of CY itself.

The mice concerned were given a single dose of 100 mg/kg CY. Whereas no gross pathology was found and histological examination revealed few pathological changes in the small intestine, measurements of mucosal architecture and electron microscopy did reveal significant abnormalities. The principal effects of CY on the mucosa were seen in the rapidly dividing crypt cells and other abnormalities were probably secondary to this action. Crypt cell inclusion bodies were seen by both light and electron microscopy only within the first 48 hours after CY, in agreement with previous studies (Waldeck 1972; Sobhon et al 1977; Hartwich et al 1978). These appeared to be contained within lysosomal bodies in crypt cells themselves, but it was not possible to identify their nature or origin. Other workers have classified these as being of nuclear material (Waldeck 1972) and it has been suggested that this represents debris of lymphoid cells or eosinophils rather than crypt epithelial cells (Sobhon et al 1972). The rapid rebound recovery of CCPR indicates that the crypt cells suffered only transient damage in these studies.

In contrast to an earlier report, inclusion bodies were not seen in villus epithelial cells (Waldeck 1972) and light microscopy showed no damage to these cells. However, mitochondrial changes were seen consistently in cells near the mouth of crypts and base of villi at all times after CY. It is difficult to assess the significance of this feature however. Mitochondrial swelling has been described in response to agents which interfere directly or indirectly with oxidative phosphorylation (King, Godman & King 1972; Porter, Mikles-Robertson, Kramer & Dane 1979) and it is conceivable that CY may have induced these alterations via its effect on DNA synthesis and hence on protein synthesis. Yet there are several pieces of evidence which argue against this. In other reports of drug-induced mitochondrial damage, the changes were consistent throughout each cell. This was not the case in my study and in addition, epithelial cells were otherwise normal. Furthermore, CY is eliminated from the mucosa within 5 days (Tew & Taylor 1977) and it would be unusual to observe direct toxicity up to 12 days. Finally, mitochondrial swelling was not reported in a study of duodenal morphology in humans given 100 mg/kg CY (Wolf, Ley & Klein 1968). It may be that the lesions observed are artefacts. Mitochondria are exquisitely sensitive to damage during fixation and processing (Toner, P.G. - personal communication) and their structure is closely related to the metabolic state of their membrane enzymes (Lehninger 1962; Hackenbrock 1966). Cells leaving the hyperactive crypts

during the recovery phase after CY will be metabolically active and it is possible that their mitochondria may be particularly fragile and sensitive to damage during fixation. This phenomenon would disappear as cells mature on the villus, and as observed here, villus cell mitochondria would appear normal. Detailed studies of mitochondrial ultrastructure and enzyme function are required to confirm the theory that these changes are secondary to a high level of proliferation during the recovery from CY.

The period of enhanced CCPR was found until 14 days after CY, during which time mitochondrial lesions were observed. Although earlier studies have identified this rebound phenomenon these were usually terminated within a few days and the prolonged nature of the recovery phase has not been appreciated (Rosenoff et al 1975; Young 1976; Ecknauer & Lohrs 1976; Sobhon et al 1977). This rebound recovery of CCPR is a well-recognised response of the mucosa to injury and has been ascribed to two different mechanisms. It may occur in response to a decrease in the functional compartment of the villus (Rijke et al 1974; Rijke & Gart 1979) or as a consequence of the action of a drug on the crypt cell compartment itself. In this case, an agent like hydroxyurea inhibits crypt cell proliferation and causes shrinkage of the crypt cell compartment. Slowly-dividing stem cells are spared by the drug and repopulate the crypt by entering a synchronous cycle of rapid cell division (Roti Roti & Dethlefsen 1975; Hanson, Henninger, Fry & Saltese 1980).

CY also spares crypt stem cells and repopulating cells show similar evidence of synchrony (Schenken, Burholt, Hagemann & Lesher 1976; Phillips, Ross & Goldstein 1979). These features support the hypothesis that the effects of CY on the mucosa are not due to a toxic action on villus epithelial cells but reflect its cytostatic action on crypt cells. In the present study, however, villus length was found to be decreased 7 days after CY, presumably secondary to the initial inhibitory effect on CCPR. The continued enhancement of CCPR after this time may then be directed at repairing this villus cell depletion. Further evidence that the consequences of CY on the mucosa are prolonged was the unusually low CCPR seen 21 days after CY. While this was not statistically significant, it may be that this is a further phase of feedback inhibition of crypt cell mitotic activity.

The findings therefore agree with previous studies which failed to find damage to mature epithelial cells after CY and indicate that changes in CCPR are responsible for the minor, morphological changes observed. In addition, no residual effects on mucosal architecture were seen at the time when OVA fed mice were sacrificed for assessment of mucosal CMI.

Effects of cyclophosphamide on the lymphoid tissues of the small intestine

In this part of my study, attention was paid particularly to the antigen-processing structures of the Peyer's Patches and to the IEL count in CY treated mice. The overall pattern

of damage to the MLN and Peyer's Patches showed selective depletion of B cell areas in agreement with previous studies of the effect of CY on lymphoid tissues (see Chapter 4).

The IEL count fell sharply 24 hours after CY but returned to control levels thereafter. This pattern has been described previously although formal IEL counts were not done (Ecknauer & Löhrs 1976). The rapid recovery of IEL is remarkable, especially in comparison with the prolonged depletion of lamina propria lymphoid cells and of other lymphoid organs. Rapidly dividing T cells are probably sensitive to CY (Kumararatne, Gagnon & Smart 1980) and the immediate fall in IEL count is consistent with the IEL being derived from a rapidly dividing pool of T cells, these precursors then recovering quickly after CY. Of interest in this respect is the fact that many IEL seen 2-4 days after CY had the appearance of recently divided cells. Finally, IEL counts were again normal at the time equivalent to that when mice in Chapter 10 were examined for mucosal CMI.

The M cells of the Peyer's Patches are thought to be specialised epithelial cells responsible for uptake of protein antigens and are probably important elements of the mucosal immune apparatus (Owen 1977). It is notable therefore that CY induced less marked mitochondrial changes in these cells than in surrounding epithelial cells. In addition, both scanning and transmission electron microscopy revealed an apparent increase in their number after CY, while the surface folds were hypertrophied in CY treated mice. I did not attempt

to do formal counts of M cells and the observations could be due either to true hyperphasia of M cells or merely to an increase in the numbers of surface projections from an unchanged number of cells. A similar increase in prominence of M cells is described in germ-free animals in whom the underlying lymphoid tissues are depleted, as they were here (Owen & Nemanic 1978). The cause of this phenomenon is unknown and clearly detailed morphological studies should yield important information on the relationship between these cells and the lymphoid elements of the intestine. If M cells are indeed epithelial in origin, my results would indicate that they are derived from a CY-insensitive precursor which may therefore be more slowly dividing than conventional enterocyte precursors. Alternatively, the M cell may be of reticuloendothelial origin, and related to the dendritic cells and Peyer's Patch macrophages which were not visibly damaged by CY. This would also explain the relative resistance of these cells to the drug.

Antigen uptake by the cyclophosphamide-treated gut

The results discussed above indicated that consistent alterations were detectable in the jejunal mucosa 48 hours after CY, at the time when mice were fed OVA in studies of intestinal immunity. However, I had not performed studies on the function of the CY-treated intestine and others had reported epithelial enzyme deficiencies (Waldeck 1972; Ecknauer 1976; Hartwich et al 1978). Although all these changes are probably related to alterations in crypt cell

proliferation rather than cytotoxicity, I felt it essential to exclude a direct effect of CY on protein uptake by the intestine.

The results of Chapter 13 indicate that CY had no overall effect on the uptake of OVA by the gut. Absolute levels of OVA in serum after feeding were similar in CY treated and normal mice, while serum from OVA fed donors had identical effects on the immune response of recipients irrespective of whether the donors had received CY or not. These results are in contrast to the decrease in protein uptake by the epithelium of the Bursa of Fabricius in CY treated chickens (Sachs et al 1979) but may be related to the apparent preservation of M cells seen in my own experiments.

In addition to an effect on the intestinal absorption of antigen, it is possible that CY may have altered the antigen-presentation function of lymphoid accessory cells in the intestine. However, CY has been reported to have only a minimal enhancing action on phagocytosis by reticuloendothelial cells (Sharbaugh 1976; Buhles & Shifrine 1978) and there are no studies on presentation of antigen by these cells after CY. It would seem unlikely that an action of this kind could explain the effects of CY intestinal immune responses to a dietary protein. Furthermore, it has recently been reported that the suppressor T cells induced after feeding OVA to mice are eliminated by 100 mg/kg CY (Hanson, D.G. personal communication). The evidence thus indicates that the effects of CY on intestinal immunity which I have described are due

to its specific inhibitory action on suppressor lymphocytes and not to non-specific toxic damage to the mucosa or GALT.

Induction of local CMI in the GALT and mucosa of orally immunised mice

In Chapters 9 and 10 it was demonstrated that under appropriate circumstances, oral immunisation of mice with a new protein antigen may induce cell mediated immunity in the intestinal mucosa and GALT. These responses were measured using the indices of mucosal CMI described in Chapter 6 and the migration inhibition assay discussed earlier, and were found to occur in the absence of systemic immunity.

Oral administration of OVA to normal mice did not induce intestinal CMI even if the animals were subsequently challenged orally with OVA for 10 days. This concurs with several previous, unpublished attempts to sensitise animals with fed antigens. In contrast, the results of Chapter 11 showed clearly that feeding OVA produced a state of systemic unresponsiveness to parenteral immunisation.

In my experiments, CMI was observed in the GALT and/or mucosa only if mice received CY 2 days before the initial feed of antigen. In addition, CY pretreatment substantially modified the tolerant state following OVA feeding. Previous reports of the induction of mucosal CMI have identified local production of lymphokines after oral immunisation with bacteria or virus (Gadol et al 1976; Frederick & Bohl 1976; O'Neill & Bienenstock 1977) and the induction of cytotoxic T cells in Peyer's Patches after feeding allogeneic cells (Kagnoff 1978c).

The evidence discussed earlier indicates that feeding proteins normally results in immunological tolerance and there is only one previous study on induction of intestinal CMI to fed protein antigens (Huntley et al 1979). In that report, MIF production by lymphocytes from MLN, spleen and lamina propria was demonstrated in normal pigs fed hapten-protein conjugates. However, the development of MIF production was somewhat variable and was found in the MLN after repeated feeding. In addition, MIF was present in some cultures from control animals. My own experiments cannot confirm the generation of local CMI in the intestine of normal mice immunised orally with protein. On the other hand, they indicate that a CY sensitive regulatory system normally prevents such responses.

Migration inhibition was fully developed in the MLN of CY treated mice within 24 hours of feeding ovalbumin and was detectable until 14 days afterwards. This represents a rapid development of sensitisation and is surprising at first sight. However, after feeding OVA, activation of suppressor and helper T cells occurs in the GALT within 24 hours (Richman, Graeff, Yarchoan & Strober 1981) and it is likely that induction of specific effector T cells may also occur very rapidly. Mucosal changes were not examined in mice challenged orally within a short time of sensitisation and it would be of obvious importance to perform parallel experiments of this type. The loss of sensitisation from the MLN 14 days after feeding is also of interest, particularly as migration inhibition and mucosal changes were observed in mice challenged 28 days after initial feeding. Loss of MLN sensitisation could reflect the

disappearance of a transient immune response or migration of committed lymphocytes from the MLN to other parts of the intestine or GALT. The ability to mount a secondary MLN response and the abrupt rather than gradual loss of sensitisation would argue against complete abrogation of intestinal immunity in these mice. It is more likely that after oral immunisation of CY treated mice, a sequence of T cell sensitisation in the GALT is followed by emigration of DTH effector cells to the mucosa. In addition, the ability to generate a secondary response is evidence for the development of memory cells during the period of sensitisation in the GALT.

When CY treated OVA fed mice were challenged orally with OVA, specific mucosal alterations were observed. This consisted of increases in CCPR, crypt depth and IEL count and were therefore identical to those found during the intestinal phase of the GvHR. These findings support the idea that CY treatment has allowed the induction of local CMI to a fed protein antigen. In addition, it is noteworthy that crypt hyperplasia and lymphocytic infiltration have not been identified as consequences of immune complex-mediated intestinal damage. In this case, ulceration and acute inflammation are found in both small intestine (Bellamy & Nielson 1974) and colon (Hodgson, Potter, Skinner & Jewell 1978). Increases in CCPR and IEL count appear to be specific to CMI reactions in the mucosa.

In these experiments, it was hoped to mimic the severe villous atrophy and crypt hyperplasia characteristic of enteropathies associated with food hypersensitivity, such as coeliac disease. The mucosal alterations observed were considerably less severe than these lesions and I considered that this may have been due to the use of too small a dose of fed OVA. However, a tenfold increase in OVA dosage had no effect on mucosal damage in CY treated mice. It is possible that the fed doses still do not approach the intake of gluten required to induce coeliac disease in the human infant. Alternatively, it might have been possible to induce more severe mucosal changes by several weeks of challenge by feeding OVA or by challenge of isolated loops of intestine to allow prolonged contact of antigen with the mucosa. Despite the absence of major intestinal damage including villous atrophy, however, the results are consistent with the hypothesis that an increased CCPR is one end of the spectrum of damage which can occur in the small intestine as a result of immune responses to dietary antigens (Ferguson & MacDonald 1977).

The results of the pilot study employing oxazolone as a fed antigen in CY treated mice were similar to those obtained with OVA. This indicates that the phenomenon is not restricted to protein antigens and confirms the ability of CY to allow the induction of intestinal CMI. In addition, the study extends an earlier report that CY pretreatment can block the induction of tolerance by feeding contact-sensitising agents

(Polak, et al 1975). It should be noted however, that in this study, alterations in CCPR and crypt depth were observed at a time when a residual effect of CY itself may have been present. These findings therefore require confirmation.

The experiments described in this thesis were based on the ability of CY to eliminate suppressor cells and reconstitution of mice with syngeneic normal thymocytes is known to restore suppressor cell function in CY treated animals (Mitsuoka et al 1976;L'age-Stehr & Diamantstein 1978). In the present study, CY treated mice given thymocytes on the day of primary feeding of OVA had mucosal changes on challenge which were identical to unreconstituted mice. While this might argue against elimination of suppressor cells by CY, it has been suggested that suppressor cells should be present in the intestine at the time of feeding antigen for effective activation (Hanson, D.G. - personal communication). In his experiments, Hanson found that oral tolerance to OVA was dependent on suppressor cell activation and that this could be abolished by prior irradiation. Unresponsiveness could be restored by transfer of normal spleen cells, but only if the cells were given at a time which allowed presumptive localisation in the intestine by the time antigen was fed. The design of my own experiment would therefore have been unable to demonstrate directly a population of CY sensitive suppressor cells responsible for preventing mucosal immunity to dietary antigens.

CY pretreatment may enhance systemic CMI without significantly altering the humoral response (Askenase et al 1975) and serum antibodies were not detectable in OVA fed mice with intestinal CMI. The results may thus reflect a preferential enhancement of CMI in the mucosa and GALT. Parallel studies employing sensitive assays for specific secretory and serum antibody are required to confirm this hypothesis for immunity to dietary antigens.

It is clear that systemic CMI did not develop at any time after oral immunisation despite the presence of CMI in the intestine and GALT. There is considerable evidence that intestinal T cells represent a pool of lymphocytes which has a separate origin and route of migration from peripheral T cells (Guy-Grand et al 1974; 1978; Cahill et al 1977; Parrott & Rose 1978). In addition, oral presentation of soluble antigen only rarely induces systemic CMI in adult animals (Goldberg et al 1971; Perrotto et al 1974). It is entirely conceivable therefore that fed protein antigens may induce CMI in the GALT with subsequent migration of gut-associated T cells in the absence of systemic sensitisation. This theory is supported by the finding that CMI may be induced in the respiratory tract in the absence of systemic CMI after inhalation of antigen (Henry & Waldman 1970). Furthermore, intestinal B lymphocytes (Craig & Cebra 1971; Guy-Grand et al 1974; Rudzik et al 1975) and secretory antibody responses (Dolezel & Bienenstock 1971a,b) form a compartment which is separate from their peripheral counterparts.

The present results are strong support for the segregation of mucosal and systemic T cells and CMI responses. It is important to reiterate however, that local CMI responses to fed proteins will only occur if the immune system has been manipulated in some way, as by CY pretreatment in my own experiments.

Induction of tolerance by feeding ovalbumin

The development of local CMI to fed OVA is one end of the spectrum of immunological consequences of feeding proteins. In Chapters 11 and 13, the effects on subsequent systemic immunity of feeding OVA were considered and a range of such effects were demonstrated. Thus, depending on the dose of fed protein used, both humoral and cell mediated immunity may be tolerised; alternatively, CMI alone may be reliably suppressed. The studies also confirmed the ability of CY to modulate the systemic immune response to ingested antigen with CMI apparently more susceptible to CY pretreatment than humoral immunity. In addition, evidence was obtained to suggest that tolerance of CMI is due to the presence of tolerogenic fragments of protein absorbed through the intestine. Serum containing these materials had no effect on humoral immunity.

Several different schedules have been used to investigate oral tolerance to ovalbumin and the present study was designed to use a single dose of OVA known to induce tolerance reliably (25 mg). In addition, a smaller dose (2 mg) which is nearer

the lower limit of tolerogenicity was also employed (Hanson et al 1977; Vaz et al 1977; Challacombe & Tomasi 1980). It should also be noted that although the optimum interval between feeding and immunisation for the demonstration of tolerance is seven days, similar unresponsiveness is found when a 14 day interval is employed as here (Vaz et al 1977; Challacombe & Tomasi 1980). The results of my experiments are therefore compatible with those of previous studies.

In addition to the discrepancy between the susceptibility of the systemic antibody and CMI response to reduction by prior feeding, it is apparent that differences exist within the humoral response itself. While it is reported that feeding OVA inhibits subsequent total serum antibody responses (Hanson et al 1977; 1979; Ngan & Kind 1978) and serum IgE responses (Ngan & Kind 1978; Vaz et al 1977), there is evidence to suggest that IgE may be more readily suppressed than IgG responses (Ngan & Kind 1978). No such evidence exists on the relative susceptibilities of the IgM and IgG phases of the serum antibody response. In this study, while the peak IgM antibody response was suppressed by feeding both 2 and 25 mg OVA, only the higher dose was effective in reducing IgG levels. Furthermore, mice receiving 2 mg OVA subsequently developed normal IgG levels despite a previously suppressed IgM response, and it is clear that IgM production may be more readily tolerised by oral antigen. In support of this, Kagnoff has shown that IgM PFC in the spleen are more susceptible to modulation by repeated feeding of SRBC (Kagnoff 1978a).

The major discordance found in my study of oral tolerance was that between systemic CMI and the IgG antibody response. The results confirmed other reports in which systemic CMI was readily tolerised by feeding OVA (Miller & Hanson 1979; Challacombe & Tomasi 1980; Titus & Chiller 1981) and indicated that CMI is more susceptible to tolerance than the humoral response. Furthermore, serum taken from OVA fed mice was effective in suppressing CMI but not humoral immunity in recipient animals. In the context of the differing effects of feeding OVA on systemic immunity, the consequences of CY pretreatment of OVA fed mice are also important. In mice fed 25 mg OVA, the similar degrees of suppression of IgM, IgG and CMI responses were returned to a level midway between control and tolerant mice by CY treatment. In mice fed 2 mg OVA however, the suppressed CMI responses were returned to control levels by CY, while the reduced IgM levels were not completely restored to normal.

The induction of tolerance by parenteral antigen may have discordant effects on subsequent CMI and humoral immunity (Neveu & Borduas 1974; Silver & Benacerraf 1974) and may involve suppressor cells (Basten et al 1974), depletion of helper T cells (Endres & Grey 1980a,b; Parks & Weigle 1980b) and B-cell inhibition (Parks & Weigle 1980b). The present results are compatible with a system of oral tolerance dependent on two or more of these mechanisms. Indeed, it has been suggested previously that oral tolerance may have multiple regulatory mechanisms (Hanson et al 1979).

While serum antibody (Kagnoff 1978a; 1980) and immune complexes (André et al 1975) have been reported to be responsible for oral tolerance to particulate antigens, it is likely that suppressor T cells induced in the spleen and GALT are responsible for oral tolerance to protein antigens (Ngan & Kind 1978; Richman et al 1978; Miller & Hanson 1979; Challacombe & Tomasi 1980; Titus & Chiller 1981). In particular, suppressor T cells are important in the reduction of CMI responses after feeding OVA (Miller & Hanson 1979; Challacombe & Tomasi 1980; Titus & Chiller 1981) and the activation of these cells by feeding OVA is inhibited by CY (Hanson, D.G. personal communication). Furthermore, the induction of tolerance by feeding contact-sensitising agents to guinea pigs is related to the appearance of suppressor cells and is abrogated by CY (Polak et al 1975; Asherson et al 1977). Thus the tolerance of CMI induced by a low dose of OVA which was fully sensitive to CY in the present study suggests that a suppressor cell system is entirely responsible for this particular unresponsive state.

The tolerance of both antibody and CMI responses induced by the larger dose of OVA was however only partially reversible by CY pretreatment. This may reflect the induction by larger doses of oral protein of tolerogenic mechanisms additional to suppressor cells. Feeding OVA to mice is known to induce defective helper T cell function in addition to simultaneous activation of suppressor T cells (Titus & Chiller 1981) and a defect of this kind may therefore be produced by larger

doses of oral protein. This mechanism is consistent with the apparent disturbance in the switch from IgM to IgG production in mice tolerised with 25 mg OVA, while a defect in an effector or accessory T cell required for expression of DTH may also account for the partial CY sensitivity of the CMI tolerance in these animals.

A further level of control may be responsible for the peculiar sensitivity of IgM synthesis to oral tolerance. It is possible that this reflects a transient inhibition of B cell function in fed mice. Indeed, although B cell priming may occur (Titus & Chiller 1981) a defect of this nature has been described after protein feeding (Vives, Parks & Weigle 1980). If this is overcome in the presence of helper T cells a mature antibody response will eventually ensue as seen in the mice fed 2 mg OVA. After feeding 25 mg OVA however, functional deletion of helper T cells would not allow a full IgG response to occur and B cell recovery may be manifest as a delayed IgM response, as was observed here. Thus, these results support the presence of several regulatory mechanisms governing systemic immune responses after feeding protein antigens.

Influence of intestinal protein handling on immune responses to dietary antigens

The results obtained in Chapter 13 indicate that antigenic material, absorbed and processed by the small intestine, is responsible for activating some of the regulatory systems discussed above.

The transfer of tolerance by serum from protein fed mice has not previously been described, although tolerogenic material has been found in the serum of mice after repeated oral doses of SRBC (André et al 1975; Kagnoff 1978a; Chalon, Milne & Vaerman 1979). In this rather different system however, it is unlikely that these findings reflect an effect of circulating antigen alone, since antibodies to SRBC (Kagnoff 1978a) and immune complexes (André et al 1975) have been described in the serum used in these experiments. More recently, a serum factor has been characterised in SRBC fed mice bearing idiotypic features similar to that found in the serum of mice tolerised parenterally with SRBC (Kagnoff 1980). These findings are likely to be due to an immune response to the oral antigen. In the present study however, serum was obtained one hour after feeding, before any immune response could occur and the results therefore reflect a property of circulating absorbed antigen.

In vitro digestion of bovine serum albumin produces protein fragments which are tolerogenic <u>in vivo</u>, possibly via suppressor cell activation (Dosa, Pesce, Ford, Muckerheide & Michael 1979). In addition, highly deaggregated protein molecules are found in serum after feeding OVA (Swarbrick 1979) and deaggregated proteins are effective in the induction of tolerance by the parenteral route (Chiller et al 1971). The results described here are therefore consistent with the hypothesis that tolerogenic materials are produced after processing of protein antigens by the gut and these are important in the induction of oral tolerance. Furthermore,

this study supports the observations that antigen processing plays an important role in both oral and systemic tolerance (Endres & Grey 1980a,b; Vives et al 1980).

Antigen-processing in the gut occurs at several different levels in addition to that associated with conventional immunological accessory cells because proteins are subjected to digestion and absorption before encountering the lymphoid cells of the gut. All these processes are therefore likely to influence the immunological consequences of feeding proteins. This has been confirmed by recent work showing that oral tolerance to OVA is abrogated if the proteolytic activity of the intestine is inhibited by a protease inhibitor given at the time of feeding OVA (Hanson, D.G. - personal communication). The exact role of other luminal and mucosal protein-handling functions remains to be clarified, and little is known of the nature of antigen-presenting cells in the gut. While it has been suggested that Peyer's Patches are deficient in these cells (Challacombe et al 1979), others have reported that this may not be the case (Levin, Rosenstreich, Wahl & Reynolds 1974). The resolution of this controversy is of obvious importance in the understanding of intestinal immunity.

In the context of my observations of depressed CMI in the recipients of serum from OVA fed mice, it is possible that tolerogenic moieties produced by the functional processes of the gut, bypass conventional processing by macrophages and thereby directly activate suppressor T cells (Feldmann &

Kontiainen 1976). That the quantity of antigen absorbed by the gut, rather than its nature could account for the tolerant state is unlikely. Intravenous or intraperitoneal injections of quantities of OVA similar to those absorbed by the gut are not effective in inducing tolerance (Hanson et al 1977; Mowat, Strobel, Pickering, Drummond & Ferguson - in preparation).

Immune responses to fed antigen are extremely complex in that feeding antigens may produce systemic immunisation or tolerance and/or local immunity in the intestinal mucosa. Induction of tolerance is perhaps one of the most important consequences of oral administration of antigen and if so it would not be surprising that several mechanisms have evolved, each with a role in producing this phenomenon. Events at the cellular level following oral administration of antigen are known to involve macrophages, B cells, helper T cells and suppressor T cells (Richman et al 1978; Miller & Hanson 1979; Vives et al 1980; Titus & Chiller 1981). Furthermore, a single feed of OVA has been shown to induce simultaneously in the GALT, both suppressor T cells for IgG antibody synthesis and helper T cells for IgA antibody synthesis (Richman et al 1981). This selective induction of regulatory T cells provides a mechanism for the concurrent development of systemic tolerance and immune exclusion of antigen due to local production of secretory antibody (Swarbrick, Stokes & Soothill 1979; Challacombe & Tomasi 1980) and is of obvious importance.

These diverse phenomena indicate a complex relationship between the antigen-processing functions of the intestine and its lymphoid tissues, the outcome of which determines the local and systemic consequences of oral immunisation with a protein antigen. However, the results of Chapters 11 and 13 indicate that systemic CMI may be regulated by a system of CY-sensitive suppressor cells in fed animals and that intestinally produced protein fragments activate these cells.

Clinical significance of mucosal CMI to dietary antigens

Several diseases cause enteropathy and malabsorption and are associated with hypersensitivity to food antigens. Of these, both coeliac disease and cow's milk protein intolerance have intestinal lesions including crypt hyperplasia (Watson & Wright 1974; Kosnai et al 1980) and lymphocytic infiltration of the epithelium (Ferguson 1974; Phillips et al 1979). Furthermore, lymphokines are produced in response to gluten by peripheral blood lymphocytes (Bullen & Losowsky 1978) and by cultured jejunum (Ferguson et al 1976) from patients with coeliac disease. It is likely that the intestinal pathology in these diseases is due to mucosal CMI to a dietary antigen.

My work, described in this thesis, indicates that after feeding antigen, systemic CMI is regulated by a suppressor cell system. Abrogation of this system by CY allows the induction of local CMI in the GALT and mucosa with consequent mucosal damage. It is possible that similar defects in

suppression may occur during early life when the body meets many new antigens via the small intestine and this offers a mechanism for the pathogenesis of food allergic diseases which damage the intestine in infancy. The precise outcome would depend on the nature of the suppressor cell defect, whether antigen-specific, immunoglobulin class-specific, transient or permanent and its time of onset. Causes for such defects in suppression might include inherited immunological deficiencies and infections or iatrogenic factors including radiotherapy and drug treatment. This kind of occurrence during the period of exposure to new dietary antigens in infancy may then predispose to potentially harmful mucosal CMI. These reactions are rare and clearly the suppressor cell system of the GALT implicated by these experiments and described by others is of considerable benefit particularly in infancy. The suppressor system of the GALT may be one of the most important homeostatic mechanisms during early life.

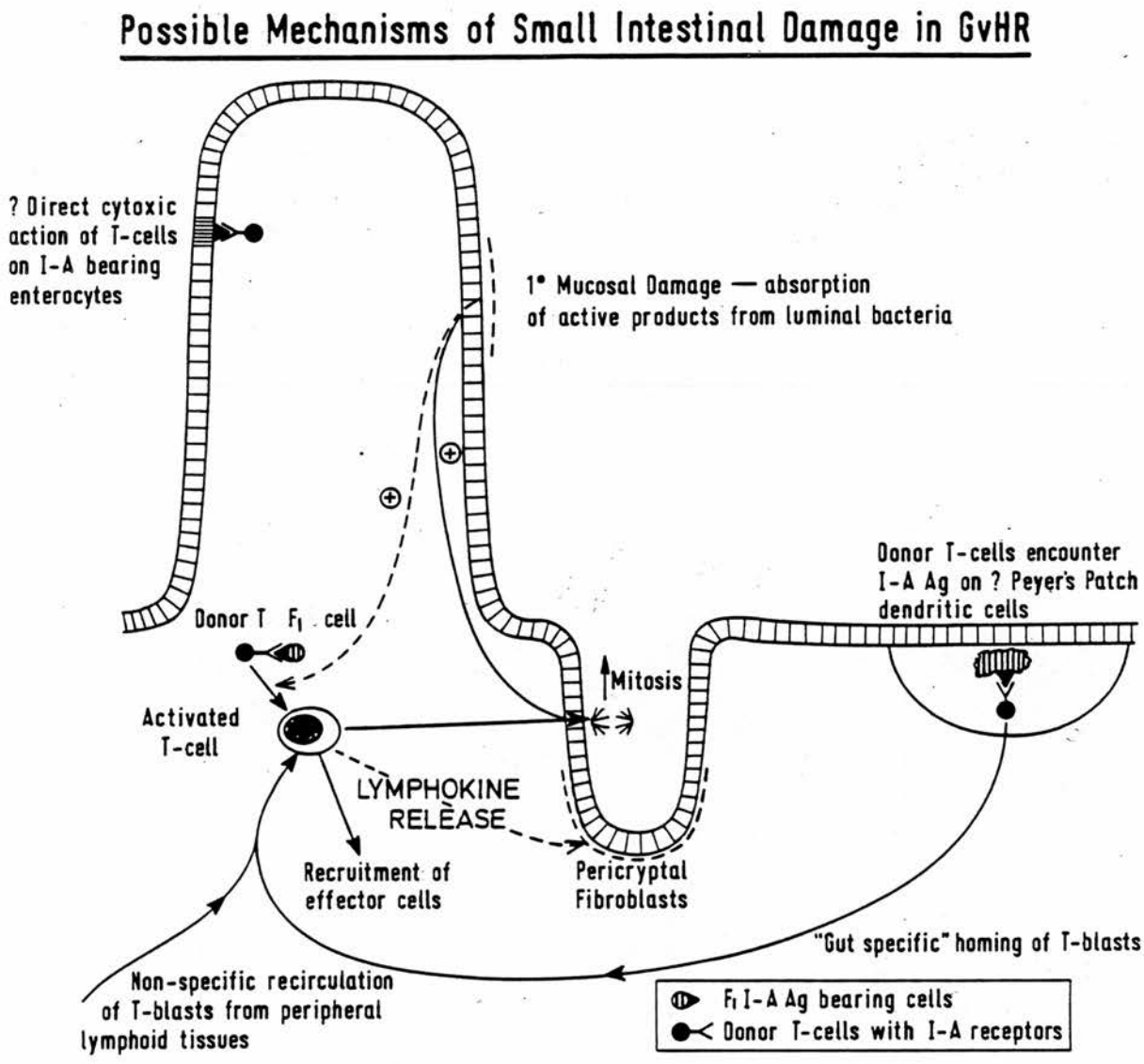

Fig. 14.1. Pathways of T cell activation and mucosal damage during the GvHR. See text for details.

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